PLASMID MAINTENANCE SYSTEM FOR ANTIGEN DELIVERY

1. BACKGROUND OF THE INVENTION

1.1 Field of the Invention

The present invention relates generally to expression plasmids stabilized by a Plasmid Maintenance System (as defined herein) capable of expressing a protein or peptide, such as an antigen for use in a live vector vaccine, and methods for making and using the stabilized plasmids. The invention optimizes the maintenance of expression plasmids at two independent levels by: (1) removing sole dependence on catalytic balanced lethal maintenance systems; and (2) incorporating a plasmid partition system to prevent random segregation of expression plasmids, thereby enhancing inheritance and stability.

1.2 Description of Related Art

Set forth below is a discussion of art relevant to the present invention.

1.2.1 Bacterial Live Vector Vaccines

Bacterial live vector vaccines deliver antigens to a host immune system by expressing the antigens from genetic material contained within a bacterial live vector. The genetic material is typically a replicon, such as a plasmid. The antigens may include a wide variety of proteins and/or peptides of bacterial, viral, parasitic or other origin.

Among the bacterial live vectors currently under investigation are attenuated enteric pathogens (e.g., *Salmonella typhi*, *Shigella*, *Vibrio cholerae*), commensals (*e.g., Lactobacillus, Streptococcus gordonii*) and licensed vaccine strains (e.g., BCG). *S. typhi* is a particularly attractive strain for human vaccination.

1.2.2 Attenuated *Salmonella typhi* as a live vector strain

S. typhi is a well-tolerated live vector that can deliver multiple unrelated immunogenic antigens to the human immune system. *S. typhi* live vectors have been shown to elicit antibodies and a cellular immune response to an expressed antigen. Examples of antigens successfully delivered by *S. typhi* include the non-toxigenic yet highly immunogenic fragment C of tetanus toxin and the malaria circumsporozoite protein from *Plasmodium falciparum*.

S. typhi is characterized by enteric routes of infection, a quality which permits oral vaccine delivery. *S. typhi* also infects monocytes and macrophages and can therefore target antigens to professional APCs.

Expression of an antigen by *S. typhi* generally requires incorporation of a recombinant plasmid encoding the antigen. Consequently, plasmid stability is a key factor in the development of high quality attenuated *S. typhi* vaccines with the ability to consistently express foreign antigens.

Attenuated *S. typhi* vaccine candidates for use in humans should possess at least two well separated and well defined mutations that independently cause attenuation, since the chance of *in vivo* reversion of such double mutants would be negligible. The attenuated vaccine candidate *S. typhi* CVD908 possesses such properties. CVD908 contains two non-reverting deletion mutations within the *aroC* and *aroD* genes. These two genes encode enzymes critical in the biosynthetic pathway leading to synthesis of chorismate, the key precursor required for synthesis of the aromatic amino acids phenylalanine, tyrosine, and tryptophan. Chorismate is also required for the synthesis of *p*-aminobenzoic acid; after its conversion to tetrahydrofolate, *p*-aminobenzoic acid is converted to the purine nucleotides ATP and GTP.

1.2.3 Plasmid Instability

Plasmidless bacterial cells tend to accumulate more rapidly than plasmid-bearing cells. One reason for this increased rate of accumulation is that the transcription and translation of plasmid genes imposes a metabolic burden which slows cell growth and gives plasmidless cells a competitive advantage. Furthermore, foreign plasmid gene products are sometimes toxic to the host cell.

Stable inheritance of plasmids is desirable in the field of attenuated bacterial live vector vaccines to ensure successful continued antigen production, as well as in commercial bioreactor operations in order to prevent bioreactor takeover by plasmidless cells.

Stable inheritance of a plasmid generally requires that: (1) the plasmid must replicate once each generation, (2) copy number deviations must be rapidly corrected before cell division, and (3) upon cell division, the products of plasmid replication must be distributed to both daughter cells.

Although chromosomal integration of foreign genes increases the stability of such sequences, the genetic manipulations involved can be difficult, and the drop in copy number of the heterologous gene often results in production of insufficient levels of heterologous antigen to ensure an optimal immune response. Introduction of heterologous genes onto multicopy plasmids maintained within a live vector strain is a natural solution to the copy number problem; genetic manipulation of such plasmids for controlled expression of such heterologous genes is straightforward. However, resulting plasmids can become unstable *in vivo*, resulting in loss of these foreign genes.

1.2.4 Plasmid Stabilization Systems

In nature bacterial plasmids are often stably maintained, even though usually present at very low copy numbers. Stable inheritance of naturally occurring lower copy number plasmids can depend on the presence of certain genetic systems which actively prevent the appearance of plasmid-free progeny. A recent review of plasmid maintenance systems can be found in Jensen *et al. Molecular Microbiol.* 17:205-210, 1995 (incorporated herein by reference).

1.2.5 Antibiotic Resistance

One means for maintaining plasmids is to provide an antibiotic resistance gene on the plasmid and to grow the cells in antibiotic-enriched media. However, this method is subject to a number of difficulties. The antibiotic resistance approach is expensive, requiring the use of costly antibiotics and, perhaps more importantly, the use of antibiotics in conjunction with *in vivo*

administration of vaccine vectors is currently discouraged by the U.S. Food and Drug Administration.

In large-scale production applications, the use of antibiotics may impose other limitations. With respect to commercial bioreactors, antibiotic resistance mechanisms can degrade the antibiotic and permit a substantial population of plasmidless cells to persist in the culture. Such plasmidless cells are unproductive and decrease the output of the bioreactor.

There is therefore a need in the art for a plasmid maintenance system specifically designed for use in bacterial live vector vaccines which does not rely on antibiotic resistance, and preferably which is also useful in commercial bioreactor applications.

1.2.6 Segregational Plasmid Maintenance Functions

Stable lower copy number plasmids typically employ a partitioning function that actively distributes plasmid copies between daughter cells. Exemplary partitioning functions include, without limitation, systems of pSC101, the F factor, the P1 prophage, and IncFII drug resistance plasmids. Such functions are referred to herein as "SEG" functions.

1.2.7 Post-Segregational Killing (PSK) Functions

Naturally occurring PSK plasmid maintenance functions typically employ a two component toxin-antitoxin system and generally operate as follows: The plasmid encodes both a toxin and an antitoxin. The antitoxins are less stable than the toxins, which tend to be quite stable. In a plasmidless daughter cell, the toxins and anti-toxins are no longer being produced; however, the less stable antitoxins quickly degrade, thereby freeing the toxin to kill the cell.

The toxins are generally small proteins and the antitoxins are either small proteins (proteic systems such as *phd-doc*) or antisense RNAs which bind to the toxin-encoding mRNAs preventing their synthesis (antisense systems such as *hok-sok*).

Balanced lethal systems discussed below in Section 1.2.7.3 are an example of an artificial PSK function.

1.2.7.1 Proteic Maintenance System: The *phd-doc* System

In proteic PSK functions, both the toxin and antitoxin are synthesized from operons in which the gene encoding the antitoxin is upstream of the gene encoding the toxin. These operons autoregulate transcription levels, and synthesis of the encoded proteins is translationally coupled. The antitoxin is generally synthesized in excess to ensure that toxin action is blocked. The unstable antitoxins are constantly degraded by host-encoded proteases, requiring constant synthesis of antitoxin to protect the cell. Upon loss of the plasmid, antitoxins are no longer produced, and the existing antitoxins rapidly degrade, permitting the toxin to kill the host cell.

The *phd-doc* system is an example of a proteic PSK function. The *phd-doc* system occurs naturally within the temperate bacteriophage P1, which lysogenizes *Escherichia coli*, as an ~ 100 kb plasmid. This maintenance locus encodes two small proteins: the toxic 126 amino acid Doc protein causes death on curing of the plasmid by an unknown mechanism, and the 73 amino acid Phd antitoxin prevents host death, presumably by binding to and blocking the action of Doc.

Phd and Doc are encoded by a single transcript in which the ATG start codon of the downstream *doc* gene overlaps by one base the TGA stop codon of the upstream *phd* gene. Expression of these two proteins is therefore translationally coupled, with Phd synthesis exceeding synthesis of the toxic Doc protein.

In addition, transcription of this operon is autoregulated at the level of transcription through the binding of a Phd-Doc protein complex to a site which blocks access of RNA polymerase to the promoter of the operon as concentrations of both proteins reach a critical level. Although Doc appears to be relatively resistant to proteolytic attack, Phd is highly susceptible to cleavage. The PSK mechanism of a plasmid-encoded *phd-doc* locus is therefore activated when bacteria spontaneously lose this resident plasmid, leading to degradation of the Phd antitoxin and subsequent activation of the Doc toxin which causes cell death.

1.2.7.2 Antisense Maintenance System: The *hok-sok* System

In antisense maintenance systems, the antitoxins are antisense RNAs that inhibit translation of toxin-encoding mRNAs. Like the antitoxin peptides, the antisense RNAs are less stable than

the toxin-encoding mRNA. Loss of the plasmid permits existing antitoxins to degrade, thereby permitting synthesis of the toxin which kills the host cell.

An example of an antisense maintenance system is the *hok-sok* system, encoded by the *parB* locus of plasmid R1. The system is comprised of three genes: *hok*, *sok* and *mok*.

Hok is a membrane-associated protein which irreversibly damages the cell membrane, killing host cells. Expression of Hok from *hok* mRNA leads to a loss of cell membrane potential, arrest of respiration, changes in cell morphology, and cell death.

The *sok* gene encodes a trans-acting RNA which blocks translation of *hok* mRNA, thereby preventing Hok killing of host cells. The *sok* RNA is less stable than *hok* mRNA and is expressed from a relatively weak promoter. (Gerdes et al. *Annu. Rev. Genet.*, 31:1-31, 1997) incorporated herein. The mechanism by which *sok* RNA blocks translation of Hok in plasmid-containing cells became apparent only after the identification of *mok* (modulation of killing), a third gene in the *parB* locus. The *mok* open reading frame overlaps with *hok*, and is necessary for expression and regulation of *hok* translation.

The *sok* antisense RNA forms a duplex with the 5' end of the *mok-hok* message rendering the *mok* ribosome binding site inaccessible to ribosomes and promoting RNase III cleavage and degradation of the mRNA. In the absence of *mok* translation, *hok* is not expressed from intact message, even though its own ribosome binding site is not directly obscured by *sok* RNA.

When a plasmid-free cell is formed, the unstable *sok* RNA decays much more rapidly than the stable *mok-hok* message. When the protection afforded by *sok* is lost, Mok and Hok are translated and the cell dies.

A limitation of the *hok-sok* system is that a significant number of plasmidless cells can arise when the *hok-sok* system is inactivated by mutations within the Hok open reading frame.

1.2.7.3 Balanced Lethal Systems

In a balanced-lethal system (a PSK function), a chromosomal gene encoding an essential structural protein or enzyme is deleted from the bacterial chromosome or is mutated such that

the gene can no longer operate. The removed or damaged gene is then replaced by a plasmid comprising a fully operating gene. Loss of the plasmid results in an insufficiency of the essential protein and the death of the plasmidless cell.

A balanced-lethal system has been successfully employed in *S. typhimurium* based on expression of the *asd* gene encoding aspartate β-semialdehyde dehydrogenase (Asd). Asd is a critical enzyme involved in the synthesis of L-aspartic-β-semialdehyde, which is a precursor essential for the synthesis of the amino acids L-threonine (and L-isoleucine), L-methionine, and L-lysine, as well as diaminopimelic acid, a key structural component essential to the formation of the cell wall in Gram-negative bacteria. Loss of plasmids encoding Asd would be lethal for any bacterium incapable of synthesizing Asd from the chromosome, and would result in lysis of the bacterium due to an inability to correctly assemble the peptidoglycan layer of its cell wall.

The *asd* system (a PSK function) has been successfully employed in attenuated *S. typhimurium*-based live vector strains for immunization of mice with a variety of procaryotic and eucaryotic antigens, including such diverse antigens as detoxified tetanus toxin fragment C and the LT enterotoxin, synthetic hepatitis B viral peptides, and gamete-specific antigens such as the human sperm antigen SP10.

Murine mucosal immunization with these live vector strains has elicited significant immune responses involving serum IgG and secretory IgA responses at mucosal surfaces.

The *asd* system has recently been introduced into attenuated *Salmonella typhi* vaccine strains in an attempt to increase the stability of plasmids expressing synthetic hepatitis B viral peptides. However, when volunteers were immunized with these live vector strains, no immune response to the foreign antigen was detected.

In fact, to date, very few reports have documented an immune response to plasmid-based expression of a foreign antigen from stabilized plasmids after human vaccination with an attenuated *S. typhi* live vector. In one report, the vaccine strain Ty21a was made auxotrophic for thymine by selecting in the presence of trimethoprim for an undefined mutation in the *thyA* gene, encoding thymidylate synthetase.

Although in some cases failure of live vector strains may have resulted from over-attenuation of the strain itself, it appears probable that current killing systems for plasmids suffer from additional limitations. In those situations where the chromosomal copy of the gene has been inactivated, rather than removed, may allow for restoration of the chromosomal copy via homologous recombination with the plasmid-borne gene copy if the bacterial strain utilized is recombination-proficient.

Balanced-lethal systems based on catalytic enzyme production are subject to a number of important deficiencies. In particular, since complementation of the chromosomal gene deletion requires only a single gene copy, it is inherently difficult to maintain more than a few copies of an expression plasmid. The plasmidless host strain must be grown on special media to chemically complement the existing metabolic deficiency.

Moreover, plasmidless cells may also benefit from "cross-feeding" effects when a diffusible growth factor is growth limiting.

There is therefore a need in the art for a Plasmid Maintenance System which is not solely reliant on a balanced lethal system, particularly for use in bacterial live vector vaccines.

2. SUMMARY OF THE INVENTION

The present invention relates generally to a stabilized expression plasmid comprising a Plasmid Maintenance System and a nucleotide sequence encoding a protein or peptide, such as a foreign antigen, and methods for making and using such stabilized expression plasmids. The Plasmid Maintenance System of the present optimizes viability by using stabilized lower copy number expression plasmids capable of expressing high levels of heterologous antigen in response to an environmental signal likely to be encountered *in vivo* after the vaccine organisms have reached an appropriate ecological niche.

In a particular aspect, the stabilized expression plasmid is employed in a *Salmonella typhi* live vector vaccine, such as the strain CVD908-*htrA*.

The invention optimizes the maintenance of expression plasmids at two independent levels by: (1) removing sole dependence on balanced lethal maintenance systems; and (2) incorporating

a plasmid partition system to prevent random segregation of expression plasmids, thereby enhancing their inheritance and stability. In one aspect of the invention, the stabilized expression plasmid is recombinantly engineered to express one or more antigens, preferably one or more Shiga toxin 2 (Stx2) antigens or substantial homologues thereof, such as Shiga toxin subunit pentamers or a genetically detoxified Stx 2.

The stabilized expression plasmid preferably comprises one or more non-catalytic plasmid maintenance functions.

In another aspect, the expression plasmid comprises a Plasmid Maintenance System which comprises at least one PSK function and at least one SEG function. For example, the Plasmid Maintenance System may comprise a two-component Plasmid Maintenance System comprising one PSK function and one SEG function. Alternatively, the Plasmid Maintenance System may comprise a three-component Plasmid Maintenance System comprising a PSK function, a SEG function and another PSK. In a preferred alternative, the Plasmid Maintenance System comprises *hok-sok* + *par* + *parA* + *phd-doc*; wherein any of the stated functions may be replaced by a substantial homologue thereof.

The Plasmid Maintenance Systems can be incorporated into multicopy expression plasmids encoding one or more proteins or peptides of interest. Such multicopy expression plasmids produce a gene dosage effect which enhances the level of expression of the protein or peptide of interest. Where the Plasmid Maintenance System is to be employed in a bacterial live vector vaccine, the protein or peptide of interest is one or more foreign antigens.

In one aspect, the expression plasmid is a vaccine expression plasmid comprising a Plasmid Maintenance System and at least one antigen, for example, at least one Shiga toxin 2 (Stx2) antigen and/or substantial homologue thereof. Where the antigen is a Shiga toxin 2 antigen, the Shiga toxin 2 antigen can, for example, be either a B subunit pentamer or a genetically detoxified Stx 2.

In another aspect the expression plasmid comprises a Plasmid Maintenance System which incorporates the *ssb* balanced lethal system and the *ssb* locus of the bacterial live vector has been inactivated using a suicide vector comprising a temperature sensitive origin of replication. In one aspect, the bacterial live vector is *S. typhi* and the suicide vector is used to inactivate the

ssb locus of *S. typhi.* In one aspect, the suicide vector is a derivative of pSC101 which carries *sacB*, described herein.

In another aspect, the present invention provides a Plasmid Maintenance System incorporating a PSK function involving a silent plasmid addiction system based on antisense RNA control mechanisms that only synthesize lethal proteins after plasmid loss has occurred.

In one aspect the expression plasmid comprises a series of expression plasmids, each comprising self-contained genetic cassettes encoding regulated expression of a heterologous antigen, an origin of replication, and a selectable marker for recovering the plasmid.

In one aspect the expression plasmid comprises a Plasmid Maintenance System which incorporates a PSK function based on the *ssb* gene. In a related aspect, mutated alleles such as *ssb-1*, described herein, are incorporated into the expression plasmids to enhance higher copy number plasmids by over-expression of SSB1-like proteins to form the required biologically active tetramers of SSB.

In another aspect, the expression plasmid comprises a promoter. The promoter is preferably an inducible promoter, such as the *ompC* promoter. In one aspect, the inducible promoter is the mutated P_{ompC1}, or the P_{ompC3} promoter described herein.

In one aspect, the expression plasmid of the present invention comprises a plasmid inheritance (or partition) locus; an origin of replication selected to provide copy number which effectively stabilizes a given antigen; a PSK function; and a nucleotide sequence encoding an antigen and a promoter which ultimately controls translation of the antigen and has a strength which is selected to improve antigen production without killing the cell.

The present invention also provides a method of using the expression plasmid comprising transforming a bacterial cell using said expression plasmid, and culturing the bacterial cell to produce the protein or peptide (e.g., the antigen), and/or administering said transformed cell or cell culture to a subject. Where the transformed bacterial cells are administered to a subject, they are administered in an amount necessary to elicit an immune response which confers immunity to the subject for the protein or peptide. The subject is preferably a human, but may also be another animal, such as a dog, horse, or chicken.

In one aspect, an expression plasmid is provided which comprises at least 3 independently functioning expression cassettes wherein one cassette encodes a protein or peptide of interest and the remaining cassettes each encode a different Plasmid Maintenance Function.

In one aspect, an expression plasmid is provided which encodes (1) a test antigen operably linked to a promoter and (2) a Plasmid Maintenance System.

In another aspect, a regulated test antigen expression cassette is provided which operates such that as induction of antigen expression is increased, a metabolic burden is placed on the bacterium which leads phenotypically to plasmid instability, i.e. a selective advantage is created for all bacteria which can spontaneously lose the offending plasmid. The test antigen can be the green fluorescent protein (GFPuv). The expression cassette encoding the test antigen can also comprise an inducible promoter, such as the *ompC* promoter, positioned such that the inducible promoter ultimately drives the translation of the test antigen.

In one aspect, a method of making an expression plasmid is provided which comprises synthesizing an expression plasmid comprising at least 3 independently functioning expression cassettes wherein one cassette encodes a protein or peptide of interest and the remaining cassettes each encode a different Plasmid Maintenance Function.

In one aspect, a method of screening Plasmid Maintenance Systems is provided comprising: providing one expression cassette which encodes a protein or peptide of interest, and at least two other expression cassettes, each encoding and capable of expressing in the host bacterial live vector a different Plasmid Maintenance Function; inserting the three expression cassettes into a single expression plasmid; transforming a bacterial live vector with the single expression plasmid; culturing the transformed bacterial live vector; and determining the rate of introduction of plasmidless cells into the culture.

In one aspect, the present invention comprises an attenuated bacterial live vector vaccine comprising an attenuated bacterial live vector which has been transformed with a stabilized expression plasmid comprising a Plasmid Maintenance System, preferably a non-catalytic plasmid maintenance system.

In one aspect, the present invention comprises an attenuated bacterial live vector vaccine comprising an attenuated bacterial live vector which has been transformed with an expression plasmid comprising a Plasmid Maintenance System which incorporates at least one PSK system and at least one SEG system. The attenuated bacterial live vector can, for example, be *S. typhi* CVD908-*htrA*.

The present invention also provides a method for vaccinating a subject comprising administering to the subject an amount of a bacterial live vector vaccine sufficient to elicit an enhanced immune response. The present invention also provides a method for preventing a disease by vaccinating a subject using an amount of such bacterial live vector sufficient to elicit a protective immune response to one or more pathogens of such disease. The subject is preferably a human but may also be another animal, such as a horse, cow or pig. For example, the present invention provides a method for preventing hemolytic uremic syndrome (HUS) caused by Shiga toxin 2-producing enterohemorrhagic *Escherichia coli* by administering to a subject an amount of a bacterial live vector transformed with a stabilized plasmid encoding at least one Shiga toxin 2 antigen.

In another aspect, the present invention provides a method for screening Plasmid Maintenance Systems for efficacy, the method comprising: providing expression plasmids comprising the Plasmid Maintenance Systems described herein and encoding for a protein or peptide of interest, said expression plasmids having copy numbers which vary from low copy number (e.g. ~5 copies per cell) to medium copy number (e.g. ~15 copies per cell) to high copy number (e.g. ~60 copies per cell); transforming bacterial live vectors with such expression plasmids; and testing for rate of introduction of plasmidless cells and/or rate of growth of plasmid-containing cells. The modified origins of replication may be origins of replication from the plasmids pSC101 (low copy number), pACYC184 (medium copy number), and pAT153 (high copy number). Independently functioning plasmid replication cassettes can be utilized which permit testing of the efficiency of one or more plasmid stabilization systems as copy number is increased.

In another aspect, the present invention provides stabilized expression plasmids for use in attenuated *S. typhi* live vectors which contain a selectable marker which can readily be replaced by a non-drug resistant locus or by a gene encoding an acceptable drug resistance marker such as *aph* encoding resistance to the aminoglycosides kanamycin and neomycin.

The Plasmid Maintenance Systems of the present invention provide improved stability of recombinant plasmids, overcoming prior art problems of plasmid instability, for example, in bioreactor and live vector vaccination uses. The plasmids of the present invention are specifically tailored for vaccine applications though such plasmids are also useful in large scale protein production.

The plasmids of the present invention are a major improvement over the prior art in that they overcome the problems associated with plasmidless takeover and plasmid instability and have wide ranging utility in fields such as commercial protein production and attenuated bacterial live vector vaccine production.

There has long been a need for a solution to the problems of plasmidless takeover and plasmid stability associated with the field of vaccine delivery and protein production. The present invention solves this long felt need.

3. DEFINITIONS

The term "Plasmid Maintenance System" ("PMS") as used herein refers to a nucleotide sequence comprising at least one post-segregational killing function ("PSK") and at least one partitioning or segregating system ("SEG"), and optionally including any other Plasmid Maintenance Function.

The term "Plasmid Maintenance Function" is used herein to refer to any plasmid-stability enhancing function associated with a PMS. The term includes both naturally-occuring nucleotide sequences encoding plasmid maintenance functions, as well as nucleotide sequences which are substantially homologous to such naturally-occurring plasmid maintenance functions and which retain the function exhibited by the corresponding naturally-occurring plasmid maintenance function.

The term "Post-Segregational Killing System" (PSK) is used herein to refer to any function which results in the death of any newly divided bacterial cell which does not inherit the plasmid of interest, and specifically includes balanced-lethal systems such as *asd* or *ssb*, proteic

systems such as *phd-doc,* and antisense systems such as *hok-sok*. The term includes both naturally-occuring nucleotide sequences encoding such PSKs, as well as nucleotide sequences which are substantially homologous to such naturally-occurring nucleotide sequences and which retain the function exhibited by the corresponding naturally-occurring nucleotide sequences.

The term "substantially homologous" or "substantial homologue," in reference to a nucleotide sequence or amino acid sequence, indicates that the nucleic acid sequence has sufficient homology as compared to a reference sequence (e.g., a native sequence) to permit the sequence to perform the same basic function as the corresponding reference sequence; a substantially homologous sequence is typically at least about 70 percent sequentially identical as compared to the reference sequence, typically at least about 85 percent sequentially identical, preferably at least about 95 percent sequentially identical, and most preferably about 96, 97, 98 or 99 percent sequentially identical, as compared to the reference sequence. It will be appreciated that throughout the specification, where reference is made to specific nucleotide sequences and/or amino acid sequences, that such nucleotide sequences and/or amino acid sequences may be replaced by substantially homologous sequences.

The terms "Segregating System" and/or "Partitioning System" (both referred to herein as "SEG") are used interchangeably herein to refer to any plasmid stability-enhancing function that operates to increase the frequency of successful delivery of a plasmid to each newly divided bacterial cell, as compared to the frequency of delivery of a corresponding plasmid without such a SEG system. SEG systems include, for example, equipartitioning systems, pair-site partitioning systems, and the *par* locus of pSC101. The term includes both naturally-occuring nucleotide sequences encoding such SEG systems, as well as nucleotide sequences which are substantially homologous to such naturally-occurring nucleotide sequences and which retain the function exhibited by the corresponding naturally-occurring nucleotide sequences.

The term "detoxified" is used herein to describe a toxin having one or more point mutations which significantly reduce the toxicity of the toxin as compared to a corresponding toxin without such point mutations.

The term "immunizingly effective" is used herein to refer to an immune response which confers immunological cellular memory upon the subject, with the effect that a secondary response (to

the same or a similar toxin) is characterized by one or more of the following characteristics: shorter lag phase in comparison to the lag phase resulting from a corresponding exposure in the absence of immunization; production of antibody which continues for a longer period than production of antibody for a corresponding exposure in the absence of such immunization; a change in the type and quality of antibody produced in comparison to the type and quality of antibody produced from such an exposure in the absence of immunization; a shift in class response, with IgG antibodies appearing in higher concentrations and with greater persistence than IgM; an increased average affinity (binding constant) of the antibodies for the antigen in comparison with the average affinity of antibodies for the antigen from such an exposure in the absence of immunization; and/or other characteristics known in the art to characterize a secondary immune response.

4. BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A-1C: Genetic maps of exemplary pGEN expression plasmids (pGEN2, pGEN3, and pGEN4) of the present invention.

Figures 2A-2D: Genetic maps of exemplary oriE1-based expression plasmids (pJN72, pJN51, pJN10, and pJN12) of the present invention.

Figure 3: Flow cytometry histograms of GFP flourescence for CVD 908-*htrA* carrying expression vectors with the *hok-sok* post-segregational killing system.

Figures 4A-4B: pGEN2 nucleotide sequence 1-4199.

Figure 5: pGEN3 nucleotide sequence 1201-2400 showing the sequence of *ori*15A.

Figure 6: pGEN4 nucleotide sequence 1201-3850 showing the sequence of *ori*101.

Figures 7A-7E: Genetic maps of exemplary ori15A-based pGEN expression plasmids (pGEN91, pGEN111, pGEN121, pGEN193, and pGEN222) of the present invention.

Figure 8: Flow cytometry histograms of GFP flourescence for expression plasmids pGEN91, pGEN111, pGEN121, pGEN193, and pGEN222.

5. DETAILED DESCRIPTION OF THE INVENTION

Bacterial live vector vaccines employ a bacterial live vector to express genes encoding protective antigens of bacterial, viral or parasitic pathogens. The bacterial protective antigens are preferably non-native to the bacterial live vector, i.e. heterologous. The bacterial live vector vaccine is administered to a host, thereby exposing the expressed antigens to the host's immune system, eliciting an immune response of appropriate character to confer immunity to the host.

In order to achieve enhanced immunogenicity, the plasmids expressing such protective antigens must be stabilized. To the inventor's knowledge, no currently available *S. typhi*-based Plasmid Maintenance System takes advantage of naturally occurring partition mechanisms known to improve the stability of multicopy plasmids in other strains.

The present invention provides a non-catalytic Plasmid Maintenance System for the stabilization of expression plasmids encoding foreign antigens in a *S. typhi* live vector vaccine strain. In one aspect the *S. typhi* strain is CVD 908-*htrA*. In another aspect, the present invention improves and/or optimizes maintenance of expression plasmids by providing Plasmid Maintenance Systems which operate at two independent levels: (1) removing sole dependence on catalytic balanced lethal maintenance systems; and (2) incorporating a plasmid partition system which will prevent random segregation of the expression plasmids, thereby enhancing their inheritance and stability. A critical reason for pursuing this particular approach is that this method of improving plasmid maintenance involves no additional manipulations of the live vector strain, and therefore can improve the immunogenicity of heterologous antigens expressed within any live vector strain.

The non-catalytic Plasmid Maintenance System of the present invention improves the stability of multicopy expression plasmids within a bacterial live vector vaccine, such as CVD908-*htrA*.

In one aspect, the present invention incorporates the naturally occurring PSK function *hok-sok* from the antibiotic-resistance factor pR1, or a substantial homologue thereof, within multicopy expression plasmids. The *hok-sok* system is a silent plasmid addiction system based on antisense RNA control mechanisms that only results in synthesis of lethal proteins after plasmid loss has occurred.

The present invention also provides a plasmid maintenance system comprising a complementation-based PSK function in which the chromosomal gene *ssb,* encoding the essential non-catalytic single-stranded binding protein (SSB) required for DNA replication, is specifically deleted and inserted within a multicopy expression plasmid.

The present invention also provides an improved Plasmid Maintenance System comprising an expression plasmid encoding at least one SEG locus and at least one PSK function.

5.1 Suicide Vectors

Heterologous antigens can be expressed within live vector strains, such as CVD908-*htrA,* from genes residing either on plasmids or integrated within the chromosome. One technique for integrating these genes into the host chromosome involves the use of temperature sensitive "suicide vectors" such as pIB307 which contains a temperature-sensitive origin of replication from pSC101 (*ori*101^{ts}). The present invention provides an improved suicide vector for use in CVD908 and CVD908-*htrA*, derived from pIB307 which allows for easier construction of mutagenesis cassettes to alter the live vector chromosome.

Integration of these suicide vectors into the chromosome by homologous recombination results from temperature inactivation of the plasmid replication protein, RepA, a protein essential to the function of *ori*101. Spontaneous resolution of the resulting unstable merodiploid intermediates is detected by counter-selection for loss of the *sacB* gene contained on the resolving suicide vector. The *sacB* gene contained on all excised plasmids encodes the levansucrase enzyme, which is lethal when expressed within the cytoplasm of enteric bacteria, including *S. typhi,* growing in the presence of sucrose. Since resolving merodiploids are selected by incubating in the presence of 10% sucrose, excised plasmids will kill host bacteria unless they cure spontaneously.

This system was successfully used to integrate a kanamycin-resistance cassette into the Δ*aroC*1019 locus of CVD908. However, these experiments were successful because the gene being mobilized into the chromosome of *S. typhi* encoded a selectable drug-resistance marker. Using these early vectors, replacing the kanamycin-resistance cassette with a non-selectable marker was not successful because, although the incoming marker could be integrated into the chromosome as a merodiploid, resolution of the merodiploid to replace the drug resistance gene was never detected.

The present invention also provides a method for using such suicide vectors to inactivate the *ssb* locus of attenuated *Salmonella typhi* strains such as CVD908-*htrA*.

The present invention allows such suicide vectors to permit efficient mobilization of genes expressing proteins or peptides of interest, such as heterologous antigens, into the chromosome of *S. typhi* CVD908-*htrA* in two stages. For example, the present inventor introduced a *sacB-aph* cassette into the Δ*aroC*1019 locus, which was then selected using kanamycin. Generation of this *S. typhi* CVD908-*htrA*Δ*aroC*1019::*sacB-aph* strain produced a valuable intermediate strain into which, in theory, any structural gene can be efficiently inserted into the *aroC* locus by marker-exchange. The *sacB* gene is used as a counter-selectable marker by passing merodiploids in the presence of 10% sucrose to select for replacement of the *sacB-aph* cassette with the incoming antigen cassette, since resolution of merodiploids in the presence of sucrose will result in loss of the *sacB* gene, in order to produce viable progeny. This intermediate strain was employed to efficiently integrate the non-toxigenic mutant LT-K63 of the *E. coli* heat-labile enterotoxin, creating CVD908Δ*aroC*1019::LT-K63.

5.2 Plasmid-Based Expression of Heterologous Antigens

Although chromosomal integration of foreign genes confers stability to such sequences, the genetic manipulations involved can be difficult, and the drop in copy number of the heterologous gene often results in production of insufficient levels of heterologous antigen to ensure an optimal immune response.

In contrast, plasmid stability is a complex phenomenon which depends on multiple factors including (1) copy number of the plasmid; (2) appropriately regulated expression of genes contained within the plasmid; and (3) selective pressure for ensuring the proper segregation and inheritance of the plasmid.

To ensure stability, plasmids must be replicated in a regulated manner to prevent their copy number from rising to lethal levels.

In addition, plasmids must segregate during the division of a growing bacterium to ensure that each daughter cell receives at least one copy of the plasmid. Segregation can be a passive, random event or an active process involving synthesis of novel proteins which aid in plasmid

segregation and inheritance. Successful inheritance of randomly segregating plasmids relies on a high enough copy number of randomly distributed plasmids within a dividing bacterium to virtually guarantee inheritance of at least one plasmid by each daughter cell.

The commonly used plasmid cloning vectors, including medium copy number pBR322 derivatives and high copy number pUC plasmids, are inherited by random segregation.

Active segregation involves the synthesis of proteins which are proposed to bind to such plasmids and further coordinate with the membranes of dividing bacteria to ensure that each daughter receives at least one plasmid copy. Plasmids employing such active partitioning systems are typically very low copy number plasmids such as the F sex factor of *E. coli* or antibiotic resistance R-factors such as pR1 and pRK2.

The present invention exploits naturally occurring SEG functions to enhance inheritance of multicopy expression plasmids, which would otherwise be inherited by random segregation, to increase the stability of these plasmids.

The present invention also takes advantage of other naturally occurring genetic systems in which daughter cells which do not successfully inherit an expression plasmid will be killed and removed from the growing population, i.e., PSK functions. The incorporation of more than one category of plasmid stabilization function is referred to herein as a Plasmid Maintenance System. For example, the incorporation of both a SEG function such as a partition locus and a PSK function into a single expression plasmid yields a Plasmid Maintenance System.

It should be noted that a gene conferring resistance to a bactericidal antibiotic, such as the *aph* gene encoding resistance to kanamycin and neomycin, is also considered a PSK function, as is the *asd*-based balanced-lethal system.

5.3 Balanced Lethal Systems

One method of ensuring the inheritance of expression plasmids involves the construction of a PSK system or a substantial homologue thereof, referred to as a balanced lethal system, for plasmids expressing heterologous antigens. In a plasmid-based balanced lethal system, plasmids replicating in the cytoplasm of the bacterium express a critical protein required by the

bacterium to grow and replicate. Loss of such plasmids removes the ability of the bacterium to express the critical protein and results in cell death.

The *asd* system has recently been introduced into attenuated *S. typhi* vaccine strains in an attempt to increase the stability of plasmids expressing synthetic hepatitis B viral peptides.

However, when volunteers were immunized with these live vector strains, no immune response to the foreign antigen was detected. See Tacket *et al.*, *Infection and Immunity*, 65:3381, 1997 (incorporated herein by reference). In fact, to date, few reports have documented an immune response to plasmid-based expression of a foreign antigen from plasmids (stabilized or otherwise) after vaccination of humans with an attenuated *S. typhi* live vector.

Although in some cases failure of live vector strains may have resulted from over-attenuation of the strain itself, the inventor's conclusion is that currently used PSK functions for plasmids suffer from additional limitations, in particular, from segregation limitations and catalytic activity limitations. The present invention provides improved expression plasmids comprising enhanced segregation capabilities by incorporating at least one partitioning system along with at least one PSK system.

5.4 Segregation Limitations

One limitation of plasmid maintenance functions such as the *asd* function (as well as the *thyA* function) is that they do not enhance the inheritance of resident plasmids, which continue to segregate randomly with or without the presence of the *asd* function. Therefore, if resident expression plasmids carrying *asd* genes are inherently unstable, they will be lost, regardless of the requirement of the bacterium for Asd.

The inherent stability of an *asd* expression plasmid can be defined by growing plasmid-bearing strains in the presence of DAP, which removes the selective pressure that ensures that all viable bacteria contain the expression plasmid. If a given plasmid is inherently unstable, it will be lost from bacteria at a high rate and such plasmidless bacteria will lyse in the absence of growth supplements; the overall result of this effect will be a population of bacteria that grows much slower than wildtype unaltered strains.

The present invention improves plasmid stability by incorporating a SEG function, such as a partition locus, or a substantial homologue of a SEG function, onto the expression plasmid to enhance the inheritance of such plasmids by actively dividing bacteria. Partition loci are naturally present on the virulence plasmids of *S. typhimurium.* Tinge and Curtiss, *Journal of Bacteriology*, 172:5266, 1990 (incorporated herein by reference) reported that such partition loci were well conserved among *S. typhimurium* virulence plasmids, and that when a 3.9 kb restriction fragment encoding this locus was introduced onto the lower copy number plasmid pACYC184 (~ 15 copies per cell), the observed plasmid stability increased from 34% plasmid-containing cells to 99% plasmid-bearing cells after 50 generations. The nucleotide sequence of this locus was later determined by Cerin and Hackett, *Plasmid*, 30:30, 1993 (incorporated herein by reference), (GenBank Accession Number M97752).

5.5 Catalytic Activity Limitations

Another potential limitation of a plasmid maintenance function such as the *asd* function (as well as the *thyA* system) is its reliance on an enzyme with catalytic activity. Given that complementation with only a single copy of the *asd* gene is sufficient to remove auxotrophy, it is not clear why all copies of a multicopy plasmid should remain stable, especially if they encode an especially problematic heterologous antigen which inhibits growth of the bacterium.

Further, although higher copy number expression plasmids may express appreciable levels of a given heterologous antigen *in vitro*, such plasmids may not be maintained at the expected copy numbers *in vivo* due to toxicity and may in fact be present at much lower copy numbers, which would be expected to reduce any observed immune response specific for the heterologous antigen. Accordingly, the present invention thus provides stably maintained low and medium copy number plasmids for expressing heterologous antigens.

5.6 The Non-Catalytic *ssb* PSK function

The potential limitation of catalytic activity associated with balanced lethal systems is addressed here through the use of plasmids expressing the single-stranded binding protein (SSB) from *S. typhi* to trans-complement an otherwise lethal mutation introduced into the chromosomal *ssb* gene. The biochemistry and metabolic roles of the *E. coli* SSB protein have been extensively reviewed in Lohman et al., *Annual Reviews in Biochemistry* 63:527, 1994 and Chase et al.,

Annual Reviews in Biochemistry 55:103, 1986 (the disclosures of which are incorporated herein by reference).

SSB is a non-catalytic 177 amino acid protein, with a relative molecular weight of 19 kDa, that binds with high affinity to single-stranded DNA (ssDNA), and plays an essential role as an accessory protein in DNA replication, recombination, and repair. The biologically relevant form of SSB involved in binding to ssDNA is a tetramer, which binds in two modes to ssDNA, intimately associating with an average of either 35 (SSB_{35}-binding mode) or 65 bases (SSB_{65}-binding mode). The specific conditions controlling the preferred mode of binding are complex and depend on the surrounding concentration of monovalent and divalent salts, pH, and temperature, as well as the amount of SSB protein present. Under given conditions, high concentrations of SSB favor the SSB_{35}-binding mode, with lower SSB concentrations favoring the SSB_{65}-mode. However, it must be emphasized that in both binding modes, the required conformation of SSB is a tetramer.

Spontaneously occurring temperature-sensitive point mutations within the *ssb* gene have now been characterized at the biochemical, physiological, and nucleotide level; one such mutant, *ssb-1,* contains the point mutation His 55 to Tyr, and has been found to be unable to assemble correctly into tetramers at non-permissive temperatures and natural expression levels. These mutant strains exhibit temperature-sensitive lethal defects in DNA replication and recombination.

The segregation frequencies of plasmids carrying *ssb* which complement chromosomal *ssb* mutations in *E. coli* bacteria were examined by Porter et al. Bio/Technology 8:47, 1990 (incorporated herein by reference). They observed that in experiments involving bioreactors, the segregation frequency in plasmid-bearing strains growing in continuous culture under non-selective conditions for 150 hours was less than 1×10^{-7}; this segregation frequency was independent of copy number, as both lower copy number pACYC184 plasmids and very high copy number pUC19 plasmids were maintained at the same frequency. However, it must be noted that the plasmids involved expressed only a drug-resistance marker in addition to the SSB protein.

The present invention provides an improved plasmid maintenance system which incorporates a partition locus such as that present on pSC101, or a substantial homologue of such partition

locus, and may also incorporate an active partitioning system, or a substantial homologue thereof, such as that described above for the virulence plasmid of *S. typhimurium*.

The present invention removes dependence on catalytic enzymes to confer plasmid stability. In one aspect, mutated alleles similar to *ssb-1* are introduced into the expression plasmids to enhance higher copy number plasmids by overexpression of SSB1-like proteins to form the required biologically active tetramers of SSB. In another aspect the present invention provides a PSK function involving a silent plasmid addiction system based on antisense RNA control mechanisms that only synthesize lethal proteins after plasmid loss has occurred.

5.7 Expression Plasmids and Self-Contained Genetic Cassettes

The present invention also comprises a series of expression plasmids which are referred to herein as pGEN plasmids. pGEN plasmids comprise self-contained genetic cassettes encoding regulated expression of a heterologous antigen, an origin of replication, and a selectable marker for recovering the plasmid. This vector series has been specifically designed to test whether any Plasmid Maintenance System can increase the stability of plasmids, for example within an attenuated *S. typhi* vaccine background.

The basic structure of these vectors is represented in Figure 1, and the composite gene sequence for the vector pGEN 2 is represented in Figure 4; Figures 5 & 6 show specific composite sequences for the origins of replication in pGEN3 and pGEN4 respectively.

It is critical to note that the pGEN plasmids are designed to comprise 3 independently functioning genetic cassettes. These cassettes have been constructed such that individual components can be optimized by replacement as necessary. Accordingly, in addition to the various Plasmid Maintenance Systems described herein, the cassettes can test other promising systems now in existence or which may become available in the future. Further, the optimized plasmid(s) can be adapted to express relevant protective heterologous antigens within attenuated vaccine strains for immunization of humans.

The pGEN plasmids provide a regulated test antigen expression cassette which operates such that as induction of antigen expression is increased, a metabolic burden is placed on the bacterium which leads phenotypically to plasmid instability, i.e. a selective advantage is created for all bacteria which can spontaneously lose the offending plasmid. Thus one aspect of the

present invention provides a conditionally unstable plasmid which can be examined for stability as plasmid maintenance systems are incorporated.

In a preferred mode, the regulated test antigen expression cassette contained within the pGEN plasmids comprises the inducible *ompC* promoter, or a substantial homologue thereof, driving expression of a detectable protein, such as the codon-optimized green fluorescent protein (GFPuv, available from Clontech), overexpression of which is toxic to *E. coli* and *S.typhi*.

The present invention also comprises a series of plasmid replicons having copy numbers which vary from low copy number (i.e., ~1 to ~10, preferably ~5 copies per cell) to medium copy number (i.e., ~11 to ~25, preferably ~15 copies per cell) to high copy number (i.e., ~26 to ~100, preferably ~60 copies per cell). To accomplish this, origins of replication from the well-characterized plasmids pSC101, pACYC184, and pAT153 have been modified using polymerase chain reaction (PCR) techniques to create independently functioning plasmid replication cassettes. These replication cassettes permit testing of the efficiency of a plasmid maintenance system as copy number is increased.

The present invention also comprises selectable expression plasmids for use in attenuated *S. typhi* live vectors. These expression plasmids contain a selectable marker which can ultimately be replaced either by a non-drug resistant locus, such as *ssb*, or by a gene encoding an acceptable drug resistance marker such as *aph* encoding resistance to the aminoglycosides kanamycin and neomycin.

To accomplish this, resistance cassettes encoding resistance to carbenicillin and tetracycline have been constructed, with transcription being efficiently terminated by an *rrnB* T1T2 terminator. A detailed description of the individual components comprising the expression and replication cassettes follows.

Specific components of the Plasmid Maintenance System can be systematically inserted into the basic expression replicons to assess any individual or synergistic influence of these functions on plasmid stability in the presence and absence of selection. For example, a post-segregational killing function (e.g., the *hok* - *sok* locus) can be inserted as an *Eco*RI - *Xba*I cassette, such that flanking transcription from surrounding loci, such as the antigen and selection cassettes, is divergent and will not significantly disturb the wild type transcription levels which control the lethality of this locus (Figure 7B, pGEN111).

Similarly, the *par* passive partition locus can be inserted as a *Bam*HI - *Bgl*II fragment between the origin of replication and selection cassettes (Figure 7C, pGEN 121). Interestingly, in the work leading to the present invention, it was observed that the orientation of the *par* locus enhances synthesis of GFPuv on solid medium when inserted in the natural orientation found within *ori*101 of pSC101; this orientation was adopted for all of the expression plasmids.

The active partitioning locus is preferably the *parA* locus, constructed as an *Xho*I - *Eco*RI cassette from the same pR1 resistance plasmid from which *hok* - *sok* was adapted. To preserve natural transcription levels and regulation within this locus, the cassette is preferably positioned within an area of the expression plasmids such that flanking transcription progresses away from *parA* (Figures 7D and 7E, pGEN193 and pGEN222).

5.8 Components of the Antigen Expression and Replication Cassettes

5.8.1 Promoter

It will be appreciated by one of skill in the art that a wide variety of components known in the art may be included in the expression cassettes of the present invention, including a wide variety of transcription signals, such as promoters and other sequences that regulate the binding of RNA polymerase to the promoter. The operation of promoters is well known in the art and is described in Doi, Regulation of Gene Expression, *Modern Microbial Genetics* pages 15-39 (1991) (the entire disclosure of which is incorporated herein by reference). The ensuing description uses the *ompC* promoter by way of example, and is not meant to delimit the invention.

The promoter is preferably an environmentally regulatable promotor controlled by a biologically relevant signal such as osmolarity. In a preferred mode, the promoter is the *ompC* promoter. The *ompC* gene encodes a porin protein which inserts as a trimer into the outer membrane of a bacterial cell. Expression and control of *ompC* is complex and has recently been reviewed in considerable detail in Pratt *et al., Molecular Microbiology* 20:911, 1996 and Egger et al., Genes to Cells 2:167, 1997 (the disclosures of which are incorporated herein by reference).

Synthesis of the OmpC protein is ultimately controlled at the level of transcription by the osmolarity of the surrounding environment such that increases in osmolarity are accompanied

by increases in the transcription of *ompC*. However, increases in osmolarity do not directly mediate increases in the transcription of *ompC*. Rather, the bacterium senses the surrounding osmolarity using a two-component signal transduction system encoded by the *ompB* operon. This operon is composed of two genes transcribed in the order *envZ-ompR*. The *envZ* gene encodes a 450 amino acid (a.a.) protein, containing two transmembrane regions, which inserts into the bacterial inner membrane (perhaps as a dimer) with an N-terminal 118 a.a. osmotic-sensing domain extending into the periplasmic space and a C-terminal 270 a.a. catalytic domain extending into the cytoplasm. The C-terminal catalytic domain possesses both kinase and phosphatase activities which are modulated by osmolarity such that as osmolarity increases, kinase activity predominates, and as osmolarity drops, phosphatase activity predominates.

EnvZ kinase activity phosphorylates aspartic acid residue 55 of the 239 a.a. cytoplasmic protein OmpR, creating OmpR-P. It is the OmpR-P modified protein which binds to the *ompC* promoter and activates transcription by RNA polymerase; therefore, as osmolarity increases, increasing kinase activity of EnvZ produces higher levels of OmpR-P, which in turn lead to greater transcription of *ompC*. OmpR-P binds to a region of the *ompC* promoter spanning bases -41 (relative to the transcriptional start site of +1) to -102, with initial binding of OmpR-P to bases -78 through -102 being followed by additional binding to bases extending to -41 as the concentration of OmpR-P increases with osmolarity. In addition, OmpR-P has been shown to bind to an AT-rich upstream region extending back to base -405 which further enhances *ompC* transcription.

In a preferred embodiment the *ompC* promoter fragment from *E. coli* spans nucleotides +70 through -389. This promoter can direct transcription within attenuated *S. typhi* strains of an antibiotic resistance gene, such as the kanamycin resistance gene in an osmotically sensitive manner. For example, our experiments have demonstrated that when the concentration of NaCl in liquid growth medium was increased from 0 mM to 300 mM, resistance to kanamycin increased from 0 μg/ml to >800 μg/ml.

5.8.2 Origin Of Replication

Due to varying degrees of toxicity associated with different heterologous antigens (i.e. higher toxicity for antigens derived from parasitic organisms such *Plasmodium falciparum* vs. virtually no toxicity for the fragment C of tetanus toxin), the present invention provides live vector vaccines which preferably express such antigens from either low or medium copy plasmids. It will be appreciated by one skilled in the art that the selection of an origin of replication will depend on the degree of toxicity, i.e., the copy number should go down as toxicity to the bacterial strain goes up. In a preferred mode, the Plasmid Maintenance System(s) used are capable of stabilizing replicons of low or medium copy numbers.

It is preferable for the origin of replication to confer an average copy number which is between about 2 and about 75. In a preferred mode the origin of replication is selected to confer an average copy number which is between about 5 and about 50. More preferably the range is from about 5 to about 30. Optimally, the range is from about 15 to about 20.

In one aspect, the origin of replication is from pSC101, conferring a copy number of approximately 5 per genome equivalent.

The *ori*E1 locus specifies synthesis of a 555 base transcript called RNA I and synthesis of a 110 base antisense RNA transcript called RNA II. As RNA I is synthesized, the 5'-proximal region of the transcript adopts a stem-loop structure composed of 3 domains which can hybridize to a complementary stem-loop structure formed by RNA II, resulting in a double stranded RNA-RNA structure forming which causes plasmid replication to abort.

As synthesis of RNA I continues, generating the full-length 555 base transcript, a rearrangement of the secondary structure of the transcript destroys the initial 3 domain stem-loop structure to form an alternate stem-loop configuration which no longer hybridizes to RNA II. Formation of this alternate structure allows the transcript to hybridize to one DNA strand of the plasmid itself, forming an RNA-DNA complex which is nicked by endogenous RNAse H to trigger synthesis of the first DNA strand of the plasmid and plasmid replication.

Plasmid replication is therefore controlled by synthesis of RNA I, which undergoes a cascade of structural configurations leading to initiation of replication. The necessary progression of the

RNA I folding cascade (and resulting replication initiation) is interrupted by competition of the domains with RNA II. This mechanism is essentially the same in plasmids containing either *ori*E1 or *ori*15A.

The reason these two types of plasmids can coexist within the same bacterium is due to sequence divergence within the region of hybridization between RNA I and RNA II, such that the RNA II from *ori*15A will not hybridize to RNA I from *ori*E1; this sequence divergence also affects the stability of the RNA I: RNA II hybrid, accounting for the differences in copy number between plasmids carrying the *ori*E1 or *ori*15A origins of replication.

The structural organization of the engineered origins of replication cassettes for pSC101 (*ori*101; ~5 copies per genome equivalent), pACYC184 (*ori*15A derivative; ~ 15 copies per genome equivalent), and pAT153 (*ori*E1 derivative; ~ 60 copies per genome equivalent) are analogous in structure and function.

5.8.3 Expressed Protein Or Peptide

When the expression cassette is used to screen Plasmid Maintenance Systems, it preferably expresses a protein or peptide with no metabolic activity. A preferred protein is the green flourescent protein (GFP) of the bioluminescent jellyfish *Aequorea victoria*, a 238 amino acid protein which undergoes a post-translational modification in which 3 internal amino acids (^{65}Ser-Tyr-Gly^{67}) are involved in a cyclization and oxidation reaction. The resulting fluorophore emits blue-green light maximally at a wavelength of 509 nm upon irradiation with long-wave ultraviolet light at a wavelength of 395 nm. In addition, fluorescence activity is remarkably constant over a wide range of pH from 5.5 - 12 and at temperatures up to 70°C.

Since GFP has no known catalytic activity, the level of observed fluorescence within individual bacteria expressing GFP can provide a direct indication of transcription levels of the *gfp* gene carried by each bacterium. Expression of the GFP protein has now been quantitated in a variety of both prokaryotic and eukaryotic cells and requires no additional cofactors or enzymes from *A. victoria*. Fluorophore formation is apparently dependent either on ubiquitous enzymes and cofactors, or is an autocatalytic event.

Individual bacteria expressing GFP can be quantitated either alone or within macrophages, epithelial cell lines, and infected animal tissues using flow cytometry. GFP fluorescence is absolutely dependent on residues 2-232 of the undenatured protein. However, fusion of unrelated biologically active protein domains to the N-terminus of GFP has still resulted in fusion proteins with the expected heterologous biological activity which continue to fluoresce as well.

It has been confirmed by sequence analysis (Clontech) that the *gfp* allele preferred here (i.e. *gfpuv*) expresses a GFP mutant (GFPuv) containing 3 amino acid substitutions (not involving the fluorophore) which increase fluorescence 18-fold over that of wildtype GFP.

In addition, 5 rarely used arginine codons have been optimized for efficient expression of GFP in *E. coli*. Since comparison of expression levels of various heterologous proteins in *E. coli* and CVD908 has demonstrated equivalent or superior expression within CVD908, it was expected that *gfpuv* will function efficiently in CVD908-*htrA*.

A coding sequence is inserted in a correct relationship to a promoter where the promoter and the coding sequence are so related that the promoter drives expression of the coding sequence, so that the encoded peptide or protein is ultimately produced. It will be understood that the coding sequence must also be in correct relationship with any other regulatory sequences which may be present.

5.8.4 Heterologous Antigens

The expression plasmids of the present invention preferably express an antigen for presentation to a host to elicit an immune response resulting in immunization and protection from disease. While Shiga toxins are presented herein as examples of antigens usefully expressed by the vaccine expression plasmids disclosed herein, the invention is broad in scope and encompasses the expression of any antigen which does not destroy the bacterial live vector and which elicits an immune response when the bacterial live vector containing said expression plasmid(s) is administered to a host, i.e., a human or other animal.

The vaccine expression plasmids provided herein are used to genetically transform attenuated bacterial strains, preferably strains used for human vaccination and most preferably used to

transform attenuated *S. typhi* vaccine strains such as CVD908-*htrA*, and preferably encode either the B subunit of Stx2 or a genetically detoxified Stx2 holotoxin.

A subset of STEC most often referred to as enterohemorrhagic *E. coli* (EHEC) are capable of causing severe clinical syndromes including hemorrhagic colitis, hemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP) in a small proportion of infected individuals, in addition to causing non-bloody diarrhea in most others.

Hemorrhagic colitis is characterized by copious bloody diarrhea, usually without fever or with only low-grade fever and a relative paucity of fecal leukocytes demonstrable in the diarrheal stools. These features differentiate hemorrhagic colitis from dysentery caused by *Shigella* which is typically scanty stools of blood and mucus, preceded by high fever and with large numbers of fecal leukocytes visible by microscopy.

HUS, a potentially fatal disease that most often affects young children but may afflict individuals of any age, is characterized by the triad of microangiopathic hemolytic anemia, thrombocytopenia and uremia. Currently in North America, HUS is the most frequent cause of acute renal failure in infants and young children. In a study by Siegler et al. of 288 patients treated for postdiarrheal HUS in Utah from 1970 - 1994, severe disease (defined as anuria lasting longer than 7 days, oliguria lasting for longer than 14 days, or extrarenal structural damage such as stroke) occurred in 25% of cases and was associated with children less than two years of age; about one third of these severe cases of HUS resulted in death (5%) or severe sequelae including end-stage renal disease (5%) or chronic brain damage (3-5%), with less severe chronic problems involving hypertension, proteinuria, or azotemia.

TTP, which most often affects adults, is characterized by neurologic complications such as stroke, in addition to thrombocytopenia, hemolytic anemia and renal disease.

By far the most common EHEC serotype is O157:H7. Nevertheless, other EHEC serotypes also cause HUS and hemorrhagic colitis, including O26:H11, O111:H8 and a number of others. EHEC strains associated with HUS always elaborate one or more Shiga toxins and carry a 60 MDa virulence plasmid. In addition, most also harbor a chromosomal pathogenicity island (so-called LEE) having a set of genes that encode the ability to attach and efface. It is well

accepted that Shiga toxins elaborated by EHEC play a key role in the pathogenesis of hemorrhagic colitis and HUS.

As described in detail below, the Shiga toxin family is comprised of two groups of toxins, Stx1 (which is essentially identical to cytotoxin/ neurotoxin/enterotoxin produced by *Shigella dysenteriae* type 1, the Shiga bacillus) and Stx2 (which is immunologically distinct from Stx1 and has several related variants). In the USA, the overwhelming majority of EHEC associated with cases of HUS express Stx2, either alone or in conjunction with Stx1.

The most important reservoir of EHEC infection are bovines. The single most important mode of transmission of EHEC to humans is via the consumption of under-cooked contaminated beef, most often ground beef. Less commonly, a variety of other food vehicles and other modes of transmission have been incriminated. Most notably, EHEC are one of the handful of bacterial enteric pathogens, which, like *Shigella*, can be transmitted by direct contact or by contact with contaminated fomites.

There is great anticipation and optimism on the part of most epidemiologists that irradiation of meat sold in the USA will drastically curtail the transmission of EHEC to humans, since it will curtail the single most important mode of transmission. Nevertheless, certain risk groups exposed to other modes of transmission of EHEC will not benefit from this intervention. For example, the exposure of abattoir workers to EHEC, an occupational hazard, occurs at a point in the meat processing cycle prior to when irradiation would be utilized. For such special groups such as these for whom risk will remain even after irradiation of meat becomes commonplace, anti-EHEC vaccines can be useful. The present invention provides vaccines against EHEC useful for the prevention of infection (in the animal reservoirs or in humans) and for preventing the severe complications of EHEC infection by stimulating neutralizing Shiga antitoxin.

Studies with attenuated *Vibrio cholerae* O1 expressing Stx1 B subunit have demonstrated the feasibility of eliciting neutralizing Shiga antitoxin by mucosal immunization with live vectors. However, since virtually all EHEC associated with HUS cases in the USA express Stx2, alone or in conjunction with Stx1, it is preferable that a vaccine for preventing the severe complications of EHEC infection via elicitation of toxin-neutralizing antibodies should stimulate anti-Stx2 as well as Stx1. It is within the broad scope of the present invention to provide a

stabilized plasmid system for expressing Stx2 antigens, alone or in conjunction with Stx1, in an attenuated *S. typhi* live vector.

Other antigens which may be suitably delivered according to the compositions and methods of the present invention include, for example, those for hepatitis B, *Haemophilus influenzae* type b, hepatitis A, acellular pertussis ($_{ac}$P), varicella, rotavirus, *Streptococcus pneumoniae* (pneumococcal), and *Neisseria meningitidis* (meningococcal). See Ellis *et al.*, *Advances in Pharm.*, 39: 393-423, 1997 (incorporated herein by reference).

In one aspect, the antigens encoded by the expression plasmids of the present invention are cancer vaccines.

In another aspect, the antigens encoded by these plasmids are designed to provoke an immune response to autoantigens, B cell receptors and/or T cell receptors which are implicated in autoimmune or immunological diseases. For example, where inappropriate immune responses are raised against body tissues or environmental antigens, the vaccines of the present invention may immunize against the autoantigens, B cell receptors and/or T cell receptors to modulate the responses and ameliorate the diseases. For example, such techniques can be efficacious in treating myasthenia gravis, lupus erythematosis, rheumatoid arthritis, multiple sclerosis, allergies and asthma.

5.8.4.1 The Shiga Toxin Family

Conradi in 1903 first reported that *S. dysenteriae* 1 produced a powerful exotoxin. Because injection of this toxin led to hind limb paralysis of rabbits it was originally called a neurotoxin. Subsequently this toxin, Shiga toxin, was shown to be lethal for certain cells in tissue culture (i.e., it was a cytotoxin). Vicari et al. and then Keusch et al. demonstrated that it also functioned as an enterotoxin.

Scientists now recognize the existence of a family of Shiga cytotoxins which inhibit protein synthesis, leading to cell death for susceptible cells. For many years after the revelation that such toxins were produced by certain *E. coli* strains in addition to the original Shiga toxin produced by *Shigella dysenteriae* type 1, the nomenclature for this family of toxins was confusing. Since early reports described the activity of these toxins on Vero cells (a cell line

derived from African green monkey kidney epithelial cells), many investigators called them verotoxins. Others referred to these toxins expressed in *E.coli* as Shiga-like toxins.

The protein toxins are collectively referred to herein as Shiga toxins (Stx), and the genes encoding these toxins are designated as *stx* with subscripts denoting the group and variant [i.e. stx_1 for the Shiga toxin produced by *E. coli* that is essentially identical to that of *Shigella dysenteriae* type 1 (*stx*), and stx_2, stx_{2c}, stx_{2d}, stx_{2e} for the antigenically distinct group of related toxins].

The structure, biochemistry and antigenicity of Shiga toxins are well described in Melton-Celsa et al., *Eschericia coli* 0157:H7 *and Other Shiga Toxin-producing E. coli Strains*, 1998; Takeda, *Bacterial Toxins and Virulence Factors in Disease*, 1995; Gyles, *Canadian J. of Microbiology*, 38:734, 1992; and O'Brien et al., *Current Topics in Microbiology and Immunology*, 180:165, 1992 (the disclosures of which are incorporated herein by reference).

These Shiga cytotoxins are composed of a single catalytic A subunit of approximately 32 kDa non-covalently associated with a pentameric receptor binding domain of approximately 7.7 kDa B subunits. These subunits are encoded by a single operon of the order *stxA-stxB*; transcription of the *stx* and stx_1 operons are iron-regulated in both *S. dysenteriae* type 1 and *E. coli*, but no environmental control signals have as yet been determined for any stx_2 operon. None of these toxins is encoded on a plasmid; rather they are phage-encoded (Stx1, Stx2, Stx2c, and Stx2d) or are chromosomally encoded (Stx, Stx2e).

As mentioned above, all members of the Shiga toxin family are cytolytic toxins which inhibit protein synthesis within susceptible cells by blocking the binding of elongation factor 1-dependent aminoacyl-tRNA to ribosomes. For all toxins identified from human infections, penetration of susceptible cells by endocytosis follows binding of the holotoxin to the necessary cell surface glycolipid receptor globotriaosyl ceramide (Gb_3), traffiking of the toxin to the Golgi apparatus and endoplasmic reticulum, followed by release into the cytoplasm. Shiga toxins are RNA *N*-glycosidases which depurinate a single adenine from the 28S RNA of the eukaryotic 60S ribosomal subunit, thus inactivating the 60S subunit and eventually leading to cell death.

There are six prototypic members of the Shiga toxin family: Stx, Stx1, Stx2, Stx2c, Stx2d, and Stx2e, which differ from one another immunologically and in toxic activity. Significant detail has

been included here to provide background for understanding the significance of point mutations discussed below, which are required for the genetically detoxified holotoxins. The members of the Shiga toxin family differ from one another in 3 fundamental ways, as recently summarized by Melton-Celsa et al., *Eschericia coli* 0157:H7 *and Other Shiga toxin-producing E. coli strains*, 1998.

(1) Immunologically: The Shiga toxin family is composed of two serogroups, Stx/Stx1 and Stx2; antisera raised against Stx/Stx1 do not neutralize members of the Stx2 serogroup, as judged by the Vero cell cytotoxicity assay.

(2) Structurally: Stx and Stx1 are essentially identical, differing in a single amino acid at position 45 of the mature A subunit, and the crystal structure for the Stx holotoxin has been solved. The prototype Stx2 is only 55% homologous to residues of the mature A subunit of Stx/Stx1 and 57% homologous to the mature B subunit, which explains why antisera raised against Stx/Stx1 do not neutralize members of the Stx2 group. Within the Stx2 group, Stx2e is most distantly related, sharing 93% amino acid homology to the mature A subunit of Stx2 and 84% homology to the mature B subunit; Stx2c and Stx2d are very similar to Stx2, sharing 99 - 100% homology in mature A subunit residues and 97% homology in mature B subunit residues.

(3) Cytotoxicity: Stx2 is among the most lethal of the Shiga toxins, with an LD_{50} for mice injected intraperitoneally of 0.5 - 2 ng. The LD_{50} for Stx1 and Stx2e is 200 - 400 ng, and 1 - 5 ng for Stx2d; however, Stx2d is unusual in that this toxin can become activated by murine intestinal mucus to increase the toxicity of the toxin, lowering the LD_{50} to 0.5 ng.

5.8.5 Site-Specific Mutagensis of Shiga Toxins

In one aspect, the invention provides a genetically detoxified Shiga toxin. The detoxification is accomplished by site-specific mutagenesis, introducing two defined and well-separated point mutations altering critical residues within the catalytic site of the A subunit. The invention also introduces two additional defined and well-separated point mutations within the B subunit to alter critical residues within the primary binding site (i.e. SITE I) residing within the cleft formed by adjacent B subunits of the holotoxin pentameric ring.

Prior attempts have been made to alter the lower affinity binding SITE II. However, this binding site has only been identified from molecular modeling studies, and is not extensively supported by mutational studies which favor SITE I binding of the Gb_3 receptor. Even if SITE II is an alternate low-affinity binding site allowing entry of our mutant holotoxin into susceptible cells, the inactivation of the catalytic domain will still prevent cell death.

Based on amino acid sequence alignments, X-ray crystallography studies, and molecular modeling studies, essential amino acids have been identified comprising the active site within the catalytic A subunit of Stx, as well as those residues comprising the binding SITE I within the B subunit pentamer of Stx/Stx1. It is the inventor's conclusion that the amino acids essential to the active site are selected from the group consisting of Tyr 77, Tyr 114, Glu 167, Arg 170, and Trp 203. The residues believed to be required for receptor binding to the clefts formed by adjacent B subunits include Lys 13, Asp 16, Asp 17, Asp 18, Thr 21, Glu 28, Phe 30, Gly 60, and Glu 65. These site predictions are consistent with functional studies and *in vivo* experiments using defined single and double mutations, within individual domains of the holotoxin, introduced by site-specific mutagenesis. A summary of such mutations is presented in Table 1. Based on these data and crystallographic predictions, it is within the broad practice of the invention to provide expression plasmids encoding Shiga toxins having two specific sets of point mutations within both the A and B subunits to create non-toxic mutant Stx2 holotoxins for use as vaccines, such as by expression within attenuated *S. typhi* live vectors such as CVD908-*htrA*.

Tabl 1. SITE-SPECIFIC MUTAGENESIS STUDIES					
SUBUNIT	TOXIN	MUTATION	DROP IN CYTOTOXICITY	DROP IN LETHALITY	NEUTRALIZING ANTIBODIES
A	Stx1	Leu201→ Val + of residues 202-213	NO cytotoxicity	-	-
	Stx1	Glu167 → Asp	10^3	-	-
	Stx1	Arg170 → Leu	10^3	-	-
	Stx2	Glu167 → Asp	10^3	-	-
	Stx2e	Glu167 → Asp	10^4	-	-
	Stx2e	Arg170 → Lys	10	-	-
	Stx2e	Glu167 → Asp Arg170 → Lys	10^4	-	-
	Stx2e	Glu167 → Gln	10^6	10^4	Y
B	Stx	Asp16 → His + Asp17 → His	NO cytotoxicity	-	-
	Stx	Arg33 → Cys	10^8	-	-
	Stx	Gly60 → Asp	10^6	-	-
	Stx1	Phe30 → Ala	10^5	10	Y
	Stx2	Ala42 → Thr	10^3-10^4	Y	Y
	Stx2	Gly59 → Asp	10^3-10^4	Y	Y

5.9 Pharmaceutical Formulations

It is contemplated that the bacterial live vector vaccines of the present invention will be administered in pharmaceutical formulations for use in vaccination of individuals, preferably humans. Such pharmaceutical formulations may include pharmaceutically effective carriers, and optionally, may include other therapeutic ingredients, such as various adjuvants known in the art.

The carrier or carriers must be pharmaceutically acceptable in the sense that they are compatible with the therapeutic ingredients and are not unduly deleterious to the recipient thereof. The therapeutic ingredient or ingredients are provided in an amount and frequency necessary to achieve the desired immunological effect.

The mode of administration and dosage forms will affect the therapeutic amounts of the compounds which are desirable and efficacious for the vaccination application. The bacterial live vector materials are delivered in an amount capable of eliciting an immune reaction in which

it is effective to increase the patient's immune response to the expressed mutant holotoxin or to other desired heterologous antigen(s). An immunizationally effective amount is an amount which confers an increased ability to prevent, delay or reduce the severity of the onset of a disease, as compared to such abilities in the absence of such immunization. It will be readily apparent to one of skill in the art that this amount will vary based on factors such as the weight and health of the recipient, the type of protein or peptide being expressed, the type of infecting organism being combatted, and the mode of administration of the compositions.

The modes of administration may comprise the use of any suitable means and/or methods for delivering the bacterial live vector vaccines to a corporeal locus of the host animal where the bacterial live vector vaccines are immunostimulatively effective.

Delivery modes may include, without limitation, parenteral administration methods, such as subcutaneous (SC) injection, intravenous (IV) injection, transdermal, intramuscular (IM), intradermal (ID), as well as non-parenteral, e.g., oral, nasal, intravaginal, pulmonary, opthalmic and/or rectal administration.

The dose rate and suitable dosage forms for the bacterial live vector vaccine compositions of the present invention may be readily determined by those of ordinary skill in the art without undue experimentation, by use of conventional antibody titer determination techniques and conventional bioefficacy/ biocompatibility protocols. Among other things, the dose rate and suitable dosage forms depend on the particular antigen employed, the desired therapeutic effect, and the desired time span of bioactivity.

The bacterial live vector vaccines of the present invention may be usefully administered to the host animal with any other suitable pharmacologically or physiologically active agents, e.g., antigenic and/or other biologically active substances.

Formulations of the present invention can be presented, for example, as discrete units such as capsules, cachets, tablets or lozenges, each containing a predetermined amount of the vector delivery structure; or as a suspension.

6. EXAMPLES

An isogenic series of expression plasmids composed of individual cassettes has been constructed for use in bacterial live vector vaccines, such as *E. coli* and *Salmonella*. With the exception of ribosomal binding sites (RBS), the key genetic loci controlling transcription initiation and termination, plasmid replication, or encoding expressed proteins are contained within defined restriction fragments, as depicted by the representative plasmid diagram of pGEN2 seen in Figure 1A. The basic structure of these expression plasmids will first be highlighted and then the data demonstrating the function of each locus within the attenuated vaccine strain CVD908-*htrA* will be summarized.

6.1 pGEN Structure

Transcription of any heterologous antigen to be expressed within CVD908-*htrA* is primarily controlled by an inducible promoter contained on an *Eco*RI - *Bgl*II cassette. Since the expression plasmids were initially modeled after pTET*nir*15, early versions carried the anaerobically-activated *nir*15 promoter (P_{nir15}). However, this promoter has been replaced with a more tightly regulated osmotically controlled promoter P_{ompC} which is easily manipulated *in vitro* by varying the concentration of NaCl.

Heterologous antigens are contained on a *Bgl*II - *Avr*II cassette, flanked by an optimized RBS at the 5'-proximal end and a *trpA* transcriptional terminator at the 3'-distal end of this cassette. The origin of replication for these expression plasmids has been designed as an *Avr*II - *Bgl*II cassette, and is protected from read-through transcription originating in flanking regions. These cassettes carry an extremely efficient derivative of the T1T2 transcriptional terminator at one terminus with the *trpA* transcriptional terminator from the heterologous antigen cassette at the opposite end of the replication cassette.

The flanking *Bgl*II and *Spe*I sites (see Fig. 2) between the replication cassette and the selection cassette are intended for insertion of a plasmid maintenance function, such as the *par* locus from pSC101. The selection cassettes contained within the plasmids are contained within *Spe*I - *Xba*I cassettes, and can, for example, be used to encode resistance to carbenicillin (the *bla* gene) or resistance to tetracycline (the *tetA* gene, see Fig. 1).

The drug resistance cassette can be replaced with the *ssb* gene encoding the essential single stranded binding protein of *Salmonella typhi* CVD908-*htrA*.

The flanking *Xba*I and *EcoR*I sites between the selection cassette and P_{ompC} are intended for insertion of additional maintenance functions, including a PSK locus such as *hok-sok (see Figs. 1 and 2)*, or an additional partition function such as the *parA* locus from pR1 (see Fig. 7).

6.2 Modified *ompC* Promoter

It was intended that any promoter controlling transcription of a heterologous gene be responsive to an environmental signal of biological relevance. For the expression plasmids described here, an *ompC* promoter cassette (P_{ompC}) from *E. coli* was used, which is induced by increases in osmolarity. Construction of this cassette was based on the published sequence of P_{ompC} published by Norioka *et al* (Norioka *et al.* 1986) and was carried out using synthetic primers to create a 459 bp *Eco*RI-*Bgl*II cassette in which the natural RBS was removed.

To confirm that this promoter was osmotically controlled within CVD 908-*htrA*, a derivative of pTET*nir*15 was constructed in which P_{nir15} - *toxC* was replaced by a cassette comprised of P_{ompC} driving expression of a promoterless *aphA-2* cassette conferring resistance to kanamycin. This plasmid, designated pK*ompC*, was introduced into CVD 908-*htrA* by electroporation, and recipients were screened for resistance to kanamycin on LB medium. The osmotically regulated expression of *aphA-2* was determined by inoculating CVD 908-*htrA*(pK*ompC*) into 50 ml of supplemented nutrient broth (NB) containing increasing concentrations of kanamycin from 0 to 300 μg/ml; a parallel set of cultures were set up with the identical ranges of kanamycin added, but also containing 10% sucrose to induce P_{ompC}. Cultures were incubated overnight at 37°C, and the $O.D._{600}$ was measured. Results are reported in the Table 2, Experiment 1.

TABLE 2 shows induction with osmolarity of the promoter P_{ompC}, controlling expression of resistance to kanamycin, within the attenuated *S. typhi* live vector CVD 908-*htrA*.

TABLE 2

EXPERIMENT 1 [1]			EXPERIMENT 2 [2]		
Concentration of kanamycin (μg/ml)	Low osmolarity ($O.D._{600}$)	10% sucrose ($O.D._{600}$)	Concentration of kanamycin (μg/ml)	Low osmolarity ($O.D._{600}$)	300mM NaCl ($O.D._{600}$)
0	0.92	0.35	0	0.95	1.04
50	0.13	0.35	200	0.04	0.99
100	0.07	0.31	400	0.02	0.96
200	0.03	0.21	600	0.01	0.92
300	0.02	0.19	800	0.01	0.92

[1]A culture of CVD908-*htrA*(pK*ompC*) was set up in LB broth supplemented with 0.0001% (w/v) 2,3- dihydroxybenzoic acid (DHB) and 50μg/ml of kanamycin, and was incubated for 16 hr at 37°C. This initial culture was then diluted 1:10 into fresh medium and incubated at 37°C for two hrs to provide a seed culture of exponentially growing bacteria. 50μl of this culture were then inoculated into 50 ml Nutrient Broth (NB) cultures supplemented with DHB as above, but with increasing concentrations of kanamycin; a parallel set of cultures were set up with the identical ranges of kanamycin added, but also containing 10% sucrose to hopefully induce P_{ompC}. Cultures were incubated overnight at 37°C, and the $O.D._{600}$ was measured.

[2]A culture of CVD908-*htrA*(pK*ompC*) in supplemented LB broth and kanamycin was incubated for 16 hr at 37°C, diluted 1:10 into fresh medium, and incubated at 37°C for two hrs to provide a seed culture of exponentially growing bacteria. 100μl aliquots of this culture were then inoculated into 50 ml NB broth cultures containing increasing concentrations of kanamycin from 200 to 800μg/ml; a parallel set of cultures were set up containing 300mM NaCl, and all cultures were incubated at 37°C for 16 hr. and the $O.D._{600}$ was measured.

Regardless of selective pressure using kanamycin, the presence of 10% sucrose had an inhibitory effect on the growth of CVD 908- *htrA*(pK*ompC*). However, the results suggested that *E. coli* P_{ompC} was osmotically controlled when driving *aphA-2* gene expression within CVD 908-*htrA*(pK*ompC*). To confirm this, CVD 908- *htrA*(pK*ompC*) was inoculated into 50 ml of supplemented NB broth, containing increasing concentrations of kanamycin from 200 to 800μg/ml; a parallel set of cultures was again set up containing 300mM NaCl to induce P_{ompC}. Cultures were incubated at 37°C for 16 hr, and results are reported in Table 2, Experiment 2. It was confirmed that P_{ompC}-driven expression of the *aphA-2* gene within CVD 908-*htrA* confers resistance to kanamycin at levels up to 800μg/ml in an osmotically regulated manner.

The *aph* gene cassette was then replaced with a 756 bp *Bgl*II - *Nhe*I cassette containing the *gfpuv* allele encoding GFPuv. During the visual screening of *E. coli* colonies sub-illuminated with ultraviolet light, one very brightly fluorescing colony and another representative fluorescent colony were chosen for further study, designated clone 1 and clone 3, respectively. Upon purification of the plasmids involved, it was determined that clone 1 contained a plasmid that no longer carried a *Bgl*II site separating P_{ompC} and *gfpuv*, while clone 3 carried the expected *Bgl*II site. We examined the induction of GFP expression when clones 1 and 3 are grown on nutrient agar in the presence or absence of NaCl, and determined by visual inspection that clone 3 displayed very little fluorescence when grown on nutrient agar containing no NaCl but fluoresced brightly when plated on nutrient agar containing 300mM NaCl (data not shown). Clone 1, however, had a higher background level of fluorescence when uninduced, but fluoresced intensely when induced with 300mM NaCl. To rule out mutations within the *gfpuv* gene which might affect fluorescence, we replaced P_{ompC} from clone 1 with P_{ompC} from clone 3, and confirmed the expected decrease in fluorescence as judged by sub- illumination (data not shown). We therefore concluded that differences in observed fluorescence were controlled by two genetically distinct versions of the P_{ompC} promoter, which we designate as P_{ompC1} (higher transcription levels with less osmotic control) and P_{ompC3} (moderate transcription levels with osmotic control similar to that observed for the P_{ompC} - *aph* cassette described above); we designate the plasmids containing these expression cassettes as pGFP*ompC1* and pGFP*ompC3*, respectively.

To quantify the differences in induced and uninduced expression of *gfpuv* controlled by P_{ompC1} and P_{ompC3}, GFPuv synthesis was monitored within both *E. coli* DH5α and *S. typhi* CVD 908-*htrA* using flow cytometry. This powerful technique has the unique advantages of allowing rapid measurement of GFPuv expression within large numbers of individual bacteria, as well as accurately determining the mean intensity of fluorescence due to GFPuv synthesis within each bacterial population analyzed. To accomplish this, pGFP*ompC1* and pGFP*ompC3* were introduced by electroporation, and colonies were isolated on supplemented 1X LB agar containing 100 μg/ml of carbenicillin grown at 30°C for 48 hr. Isolated colonies were then grown up and cultures frozen down as master stocks. Fresh colonies were then inoculated into either supplemented nutrient broth or supplemented nutrient broth containing 150 mM NaCl, and grown at 37°C/250 rpm for 24 hr; the difference in $O.D._{600}$ for any culture was never greater than 0.07. Induction of expression of *gfpuv*, controlled by P_{ompC1} and P_{ompC3}, was analyzed by flow cytometry, and results are presented in Table 3.

TABLE 3 shows a comparison of induction of P_{ompC1} and P_{ompC3}, controlling expression of GFPuv, within the host strains *E. coli* DH5α and CVD 908-*htrA*.[1]

TABLE 3

STRAIN	Low osmolarity ($O.D._{600}$)	Mean Fluorescence Intensity	150mM NaCl ($O.D._{600}$)	Mean Fluorescence Intensity	Induction Ratio[2]
DH5α	0.61	0.28	0.95	0.29	NA[3]
DH5α (pGFP*ompC1*)	0.56	4.45	0.72	7.69	1.7
DH5α (pGFP*ompC3*)	0.58	1.77	0.73	4.21	2.4
CVD 908-*htrA*	0.58	0.27	0.65	0.26	NA
CVD 908-*htrA* (pGFP*ompC1*)	0.60	5.37	0.54	23.4	4.4
CVD 908-*htrA* (pGFP*ompC3*)	0.54	2.56	0.53	17.1	6.7

[1]All strains were streaked from frozen master stocks onto 2X LB agar supplemented with DHB and 50 μg/ml of carbenicillin, and incubated for 36 hr at 30°C. Isolated colonies were pooled into 300 μl of NB broth supplemented with DHB and carbenicillin, from which 25 μl were inoculated into 25 ml supplemented NB broth, with and without 150 mM NaCl, and incubated at 37°C, 250 rpm for 24 hr. Bacteria were then pelleted, resuspended in 1 ml PBS pH 7.4, and then diluted 1:1000 into PBS for analysis by flow cytometry.

[2]Defined as the ratio of mean fluorescent intensity measured after induction with 150 mM NaCl, divided by basal level of mean fluorescent intensity measured at low osmolarity.

[3]NA = not applicable.

The basal level of expression for the P_{ompC1} -*gfpuv* cassette is 2.5 times higher than for the P_{ompC3} -*gfpuv* cassette, when expressed in DH5α, and 2.1 times higher when expressed within CVD 908- *htrA*; however, the basal level of fluorescence detected for synthesis of GFPuv never exceeded a mean fluorescent intensity of 5.37, regardless of host background. If we define induction ratio as the ratio of mean fluorescent intensity measured after induction, divided by basal level of mean fluorescent intensity, it was observed that when induced with 150 mM NaCl, P_{ompC1} and P_{ompC3} displayed within DH5α induction ratios of 1.7 and 2.4 respectively. Surprisingly, the induction ratio for P_{ompC1} when measured in CVD 908-*htrA* was 4.4, and

produced a maximum mean fluorescence intensity of 23.4 for these experiments. Although the induction ratio for P_{ompC3} within CVD 908-*htrA* was 6.7, the mean fluorescence intensity of 17.1 was lower than measured for P_{ompC1}. Based on these data, it appears that P_{ompC1} is the strongest and yet osmotically controlled of the two *ompC* promoters. P_{ompC1} was therefore chosen for synthesis of the widest possible range of heterologous test antigen to examine the effects of such synthesis on plasmid stability.

These data clearly show that when driving expression of *gfpuv* within the live vector strain CVD 908-*htrA*, P_{ompC1} and P_{ompC3} are inducible with increasing osmolarity, although the basal level of transcription is still noteworthy in both cases. The results observed under conditions of low osmolarity further support our observations using solid media that P_{ompC1} drives higher heterologous antigen expression than P_{ompC3}. Since P_{ompC3} was noted to possess the intended 3'- terminal *Bgl*II site, which was not detected for P_{ompC1}, we determined the nucleotide sequence for P_{ompC1} to perhaps detect point mutation(s) which might explain the strength of P_{ompC1}. The only differences identified were located at the 3'-terminus of the cassette. The intended sequence within this region was 5'-...catataacAGATCTtaatcatccacAGGAGGatatctgATG-3' (from left to right, upper case denotes the *Bgl*II site, ribosome binding site, and GFPuv start codon respectively); the actual sequence proved to be 5'-...catataacAGATC**GATCT**taa**A**catccacAGGAGG**A**t**A**tctgATG-3 (inserted or changed bases denoted with underlined bold upper case). These changes detected within the *ompC1* promoter sequence are apparently responsible for increasing the observed strength of P_{ompC1} by an unknown mechanism, since neither the basic *ompC* promoter sequence, nor the optimized ribosome binding site have been spontaneously altered.

6.3 Origins of Replication and Selection Cassettes

The success of expressing potentially toxic or otherwise problematic heterologous antigens within CVD908-*htrA* depends on the copy number of the expression plasmid. In addition, observed immune responses to a given heterologous antigen are affected by the copy number of the gene(s) encoding the antigen, with chromosomally expressed antigens eliciting poorer immune responses when compared to plasmid-based expression.

An optimized immune response will depend on multicopy plasmid-based expression of the heterologous antigen(s) from plasmids with the appropriate copy number.

Since the appropriate copy number for a given heterologous gene cannot be known *a priori*, the present invention provides a set of expression plasmids which contain the origins of replication *ori*E1 (amplified from pAT153; copy number ~60), *ori*15A (amplified from pACYC184; copy number ~15), and *ori*101 (amplified from pSC101; copy number ~5). These self-contained replication cassettes are all carried on *Bgl*II - *Bam*HI fragments, and are depicted for a set of 3 tetracycline-resistance expression plasmids shown in Figures 1A-1C.

Expression of the P_{ompC1}-controlled *gfpuv* expression cassette contained on these expression plasmids was analyzed using flow cytometry. These experiments were designed to detect whether differences in the level of observed fluorescence could be correlated with the expected copy number of a given expression plasmid. CVD908-*htrA* strains carrying pGEN2, pGEN3, and pGEN4 were streaked onto the rich medium SuperAgar supplemented with DHB and 20 µg/ml tetracycline where appropriate. SuperAgar was used because it is a very rich medium (3X LB agar). Plates were incubated at 30°C to reduce the toxicity of GFP synthesis and allow bacteria to grow luxuriously on the plates. Isolated colonies were then inoculated into 45 ml of SuperBroth supplemented with DHB and 20 µg/ml tetracycline where appropriate, and incubated at 37°C for 16 hr. Bacteria were concentrated by centrifugation and resuspended in 1 ml of sterile PBS, pH=7.4, and diluted 1:100 in PBS, pH=7.4 prior to FACS analysis. Bacteria were analyzed by flow cytometry, as described above, for two independent growth experiments, and results are displayed in Table 4 at the end of this section.

These data support the conclusion that overexpression of GFPuv within CVD908-*htrA* is toxic to the bacteria. As the theoretical copy number increases for the plasmids pGEN4, pGEN3, and pGEN2 expressing GFPuv under identical growth conditions from the identical P_{ompC1} promoter, the percentage of the growing population which fluoresces declines. It is expected that the "dim" bacteria are not viable bacteria and may no longer contain the expression plasmid, since these cultures were grown in the presence of 20 µg/ml tetracycline. It is noted, however, that when streaked onto solid medium and grown at 37°C for 24 - 36 hr, CVD908-*htrA*(pGEN2) grows poorly and fails to produce isolated colonies, while CVD908-*htrA*(pGEN3) and CVD908-*htrA*(pGEN4) grow quite well and produce intensely fluorescing isolated colonies.

GFPuv is employed herein as representative of other problematic heterologous antigens which would be of interest to include in a bacterial live vector, such as the *S. typhi*-based live vector;

however, it will be appreciated that GFPuv can be replaced by any non-metabolic protein or peptide antigen.

The data above show that although use of medium-copy expression plasmids containing *ori*E1 replicons can be of use in expression of some antigens, expression of antigens of higher toxicity will be more successfully expressed from lower copy number plasmids which employ origins of replication yielding average copy numbers between 2 and 30, such as *ori*101 or *ori*15A origins of replication.

Table 4

Strain	Experiment 1				Experiment 2			
	Percent Dim Bacteria	Mean Fluorescence Of Dim Bacteria (Relative Units)	Percent Fluorescing Bacteria	Mean Fluorescence (Relative Units)	Percent Dim Bacteria	Mean Fluorescence Of Dim Bacteria (Relative Units)	Percent Fluorescing Bacteria	Mean Fluorescence (Relative Units)
CVD908-*htrA*	100	0.6	0	0	100	0.3	0	0
CVD908-*htrA*(pGEN2)	19.9	0.1	80.1	38.5	37.2	0.3	62.8	10.1
CVD908-*htrA*(pGEN3)	17.1	0.1	82.9	28.1	4.9	0.2	95.1	8.28
CVD908-*htrA*(pGEN4)	12.1	0.1	88.0	22.4	9.4	0.3	90.6	4.25

6.4 The *hok-sok* Antisense Post-Segregational Killing Locus

Using the polymerase chain reaction, the *hok-sok* PSK genes were amplified using the multiple antibiotic resistance R-plasmid pR1 as the template in these reactions. All initial attempts to clone this locus onto either high or medium copy number plasmids were unsuccessful. In order to directly select for the *hok-sok* locus during subcloning, a set of primers was designed for use in overlapping PCR reactions such that the final product was a fragment containing a genetic fusion of the *hok-sok* locus from pR1 and a promoterless *tetA* gene from pBR322 encoding resistance to tetracycline. This cassette was engineered such that transcription of the *hok* gene would continue into *tetA*; the two loci within this cassette were separated by an *Xba*I restriction site for future manipulations.

Construction of this cassette not only allowed for direct selection of the *hok-sok* locus, but also allowed for confirmation that the PSK function would operate in *S. typhi* CVD908-*htrA*. After electroporation of plasmids carrying the cassette into CVD908-*htrA*, transformants could be selected using tetracycline. Successful recovery of isolated colonies indicates successful synthesis of the *hok* - *tetA* mRNA, and successful synthesis of the antisense *sok* RNA to prevent translation and synthesis of Hok, which would kill the bacteria. Recovery of the *hok-sok-tetA* cassette then became straightforward, and was easily incorporated into our expression plasmids to create the selectable marker cassette of the plasmids pGEN2, pGEN3, and pGEN4 depicted in Figures 1A-1C.

Experiments were then initiated to determine the effect of the *hok-sok* PSK function on the stability of expression plasmids containing *ori*E1 and the resistance marker *bla* encoding β-lactamase which confers resistance to carbenicillin. The *hok-sok* cassette was inserted into the pAT153-based expression plasmid pTET*nir*15, in which the P*nir*15-*toxC* heterologous antigen cassette was replaced with our P_{ompC1}-*gfpuv* cassette, creating the plasmids pJN72 (without *hok-sok*) and pJN51 (with *hok-sok*). An additional set of plasmids was created by replacing P_{ompC1} with the weaker promoter P_{ompC3}, creating pJN10 and pJN12; the structures of these four isogenic plasmids are represented in Figure 2. CVD908-*htrA* strains carrying either pJN72, pJN51, pJN10, or pJN12 were streaked onto the rich medium SuperAgar supplemented with DHB and 100 μg/ml carbenicillin, and plates were incubated as above for the pGEN plasmids at 30°C to reduce the toxicity of GFPuv synthesis and allow bacteria to grow luxuriously on the plates.

Isolated colonies were then inoculated into 45 ml of Super broth supplemented with DHB and 100 μg/ml carbenicillin and grown at 37°C for 24 hours for analysis by flow cytometry of fluorescence. A second independent experiment was carried out exactly as the first, except isolated colonies were suspended in 500 μl of Super broth and 250 μl each inoculated into 45 ml paired Super broth cultures with or without 300 mM NaCl added to induce the P_{ompC}-*gfpuv* cassettes; cultures were incubated at 37°C for 48 hrs and again analyzed by flow cytometry; and results for both experiments are displayed in Table 5. Fluorescence histograms for uninduced and induced expression plasmids from experiment 2 are represented in Figures 3A-3H.

Table 5

Experiment 1					Experiment 2					
Strain	Percent Dim Bacteria	Mean Fluorescence Of Dim Bacteria	Percent Fluorescing Bacteria	Mean Fluorescence	$O.D._{600}$	+/- 300 mM NaCl	% Dim Bacteria	Mean Fluorescence Dim Bacteria	% Fluorescing Bacteria	Mean Fluorescence
CVD908-*htrA*	100	0.3			0.73	-	100	0.3	0	0
CVD908-*htrA*(pJN72)	3.1	0.2	96.9	10.2	0.75	-	2.3	0.3	97.7	11.7
					0.89	+	22.2	0.3	77.8	22.5
CVD908-*htrA*(pJN51)	58.1	0.3	41.9	6.29	0.62	-	56.3	0.3	43.7	18.4
					0.82	+	95.4	0.3	4.6	21.0
CVD908-*htrA*(pJN10)	5.4	0.2	94.6	7.43	0.72	-	1.7	0.3	98.3	8.3
					0.96	+	29.9	0.3	70.1	19.8
CVD908-*htrA*(pJN12)	18.9	0.2	81.1	6.60	0.47	-	45.2	0.3	54.8	16.4
					0.68	+	95.6	0.3	4.4	13.2

These flow cytometry results can be explained as follows: expression of GFPuv (or other potentially detrimental heterologous antigen) from a multicopy expression plasmid such as pJN72 increases the metabolic stress on the CVD 908-*htrA*(pJN72) live vector, and increases plasmid instability in the absence of selection. Since the selectable marker of the expression plasmid encodes the secreted enzyme β-lactamase, then as time increases the concentration of carbenicillin in the surrounding medium declines, selective pressure decreases, and the frequency of plasmid loss increases; however, since multicopy plasmids are involved, relatively few bacteria succeed in losing all resident plasmids, but the average copy number of pJN72 per bacterium drops.

Quantitation by flow cytometry of GFPuv production for an uninduced population of healthy growing CVD 908-*htrA*(pJN72) indicates that the majority of bacteria express GFPuv and few non-fluorescing cells are detected (Figure 3A). However, increasing production of GFPuv by induction of the P_{ompC1}-*gfpuv* cassette increases the metabolic stress on CVD 908-*htrA*(pJN72), and although the production of GFP doubles, the percentage of non-fluorescent bacteria increases as more plasmids are lost from the population (Figure 3B).

In a similar population of growing CVD 908-*htrA*(pJN51), each bacterium carries multicopy plasmids encoding both GFPuv and a PSK function. The frequency of plasmid loss for pJN51 remains the same as for pJN72, but in this case as individual bacteria lose copies of the

expression plasmid, the 1:1 stoichiometry between the mRNA levels of *hok* and *sok* is disturbed, and production of Hok leads to cell death; therefore, the only CVD 908-*htrA*(pJN51) bacteria that will grow rapidly will be those which retain all of their expression plasmids. Accordingly, it is not surprising that quantitation by flow cytometry of GFPuv production for an uninduced population of healthy growing CVD 908-*htrA*(pJN51) now detects a population of fluorescing bacteria which displays levels of GFPuv fluorescence equivalent to those observed for CVD 908-*htrA*(pJN72) grown under inducing conditions (Figure 3C vs Figure 3B); however, the percentage of non-fluorescing bacteria rises to over half the overall population of organisms.

Increasing production of GFPuv in this population by induction of the P_{ompC1}-*gfpuv* cassette in CVD 908-*htrA*(pJN51) again increases the metabolic stress on the live vector, but now the percentage of non-fluorescent bacteria almost completely overtakes the few fluorescing bacteria as many plasmids are presumably lost from the population and bacteria are killed (Figure 2D).

One would expect that if a weaker promoter is used to control expression of GFPuv, the overall fluorescence of the population would be decreased (compared to that observed for a similar population of organisms grown with a strong promoter expressing GFPuv under identical conditions), and the percentage of non-fluorescent bacteria should drop due to the overall drop in GFPuv synthesis. However, as seen in Figures 3E-3H, use of the weaker P_{ompC3}-*gfpuv* cassette did not significantly improve the viability of induced bacteria carrying a killing system, even though overall expression of GFPuv was reduced.

It is concluded that in order to maximize the percentage of a population of live vectors expressing the heterologous antigen of choice, it is not sufficient only to incorporate a PSK function into a given expression plasmid, whether it be a drug resistance marker, the *asd* system, an alternate *ssb* system, or the *hok* - *sok* killing system. In addition to optimizing copy number and expression levels, the segregation frequencies of these plasmids must also be improved to ensure that each daughter cell in an actively growing population will inherit at least one expression plasmid and those that do not will be killed and removed from the population. It is therefore within the scope of the present invention to provide an expression plasmid having a PSK function and further having optimized copy number and/or expression levels, coupled with incorporation of one or more SEG functions.

6.5 Complementation-based killing system

It is also within the broad scope of the present invention to provide an expression plasmid comprising a complementation-based killing system, for example, a system involving the deletion of the chromosomal *ssb* locus of CVD908-*htrA* by homologous recombination, and trans-complementation of this lesion using multicopy plasmids carrying functional *ssb*.

To carry out such constructions requires cloning the relevant section of the *S. typhi* chromosome encompassing the *ssb* gene and flanking sequences, into which specific deletions can be introduced for chromosomal mutagensis.

Since our original submission, substantial progress has been made in the sequenceing of the *Salmonella typhi* chromosome at the Sanger Centre in London. The Sanger Centre is a genome research center set up in 1992 by the Wellcome Trust and the Medical Research Council in order to further our knowledge of genomes. Among other projects, the Sanger Centre is sequencing the 4.5 Mb genome of *S. typhi*, in collaboration with Gordon Dougan of the Department of Biochemistry, Imperial College, London. They are sequencing strain CT18, a highly pathogenic, multiple drug resistant strain isolated from a typhoid patient in Cho Quan Hospital, Ho Chi Minh City, Vietnam. This strain is known to harbor pVN100 (a 130kb multidrug resistance plasmid) and a cryptic 80kb plasmid. The genome is being sequenced by a whole genome shot gun approach using a 2 kb pUC library, generated inhouse from chromosomal DNA supplied by Prof. Dougan's lab. Each insert is being sequenced once from each end. The shotgun phase is now complete, and finishing has begun. At present there are 60 contigs over 1kb in the database; a total of 5.106 Mb of sequence assembled from 87,331 reads.

Based on updated results posted October 4, 1999, we have identified Contig 343, which contains the *S. typhi ssb* locus and critical flanking sequences within a 205,199 bp region. We have designed primers 1 and 4 (listed below) to amplify by PCR a 3535 bp fragment of the *S. typhi* chromosome in which the *ssb* locus is flanked by 1.5 kb of chromosomal sequence; this flanking symmetry is required for optimal crossover frequenceis to introduce the counter-selectable *sacB – neo* cassette and replace *ssb*. Using the methodology previously filed, we will use primers 1 and 2 to engineer a 5'-proximal 1.5 kb *Eco* RI – *Xma* I cassette, upstream of *ssb*. Primers 3 and 4 will be used to generate the 3'-distal 1.5 kb *Xma* I – *Eco* RI cassette, downstream of *ssb*; both 1.5 kb cassettes will be ligated together, forming the 3 kb *Eco* RI fragment containing a unique *Xma* I site exactly in the middle of the cassette. The *sacB – neo*

cassette can now easily be inserted into the *Xma* I site, to complete construction of the mutagenesis cassette to be inserted into pCON (previously described in our first filing). The required complementing *ssb-1* cassette will be constructed using primers 5 and 6 as a *Nhe* I cassette for replacement of drug resistance markers within the *Xba I – Spe* I cassettes of pGEN 211, pGEN 222, pGEN 206, or any later version of the expression plasmids detailed herein.

PRIMER 1:
5' – gaattcGCGCGCTTCGCGATTCAGTCGCGTTCCTTCACA
GCTGGCGCAGGGGCGATTACTGATGAA – 3'

PRIMER 2:
5' – cccggGAGTCTCCTGAATACGTTTCATAAATAGTGTAA
ACGCGTGAGTGTACCATTTCCACGTAGC – 3'

PRIMER 3:
5' – cccggGTAAAAAACTCAAAGCGTTATTTGCATTTTCGC
TATAGTTCTCGTCTGCTGAAATGCCTGGTGT – 3'

PRIMER 4:
5' – gaattcCATTTCTATCAATAAATTACTATTAGTTTTGTCT
TCTAACCAAGCCTCTATTTTATGAGTATCCTCTTCAG – 3'

PRIMER 5:
5' – gctagcATGGCCAGCAGAGGCGTAAACAAGGTGATTCT
CGTTGGTAATCTGGGCCAGGACCCGGAAGTACGC – 3'

PRIMER 6:
5' – gctagcTCAGAACGGAATGTCGTCGTCAAAATCCATTG
GCGGTTCGTTAGACGGCGCTGGCGCG – 3'

6.6 Stability of Expression Plasmids in the Absence of Selection

In order to develop a non-catalytic plasmid maintenance system to enhance the stability of multicopy expression plasmids encoding foreign antigens within CVD 908-*htrA*, experiments were initiated to monitor plasmid stability by quantitating expression of GFPuv by flow cytometry when strains were passaged in the absence of antibiotic selection. These experiments were designed to address 3 fundamental questions: 1] What is the effect of the induction level of P_{ompC1} on the stability of plasmids encoding a heterologous antigen such as GFPuv? 2] What is the effect of copy number on the stability of plasmids expressing GFPuv? 3] How do the *hok - sok*, *par*, and *parA* maintenance functions affect plasmid retention, both as individual components and synergistically?

Initial flow cytometry experiments were carried out in which CVD 908-*htrA* carried replicons with either the *ori*E1, *ori*15A, or *ori*101 origin of replication. It was quickly determined that replicons carrying the higher copy number *ori*E1 origins were very unstable, even when strains were grown in the presence of antibiotic selection. Flow cytometry results indicated that even when cultured in the presence of carbenicillin, the percentage of the bacterial populations no longer expressing detectable GFPuv ranged from approximately 50 % for pGEN71 (carrying *hok* - *sok*) and pGEN84 (*hok* - *sok* + *par*) to 62 % for pGEN211 (*hok* - *sok* + *par* + *parA*). Since replicons carrying an *ori*E1 origin clearly did not allow for optimal synthesis of the heterologous GFPuv test antigen within the majority of a growing population of live vector bacteria, this series of expression plasmids was not examined further.

CVD 908-*htrA* carrying expression plasmids with an *ori*15A origin were then examined. Strains were inoculated into 25 ml cultures of 1X LB + DHB (no antibiotic selection) containing either 50 mM, 150 mM, or 300 mM NaCl. Cultures were incubated for 24 hr at 37°C/250 rpm, diluted 1:1000 into fresh medium of identical osmolarity, and incubated for another 24 hr; samples from all cultures were analyzed for levels of GFPuv synthesis by flow cytometry. Results for the first passage in the absence of selection are listed in Table 6, and the histograms representing these data are shown in Figure 8.

TABLE 6 shows stability within CVD 908-*htrA* of *ori*15A replicons, containing plasmid maintenance systems of increasing complexity, grown without selection and in the presence of increasing osmolarity.[1]

TABLE 6

STRAIN[2]	50 mM NaCl			150 mM NaCl			300 mM NaCl		
	O.D.$_{600}$	Percent Fluorescing Bacteria	Mean Fluorescence Intensity	O.D.$_{600}$	Percent Fluorescing Bacteria	Mean Fluorescence Intensity	O.D.$_{600}$	Percent Fluorescing Bacteria	Mean Fluorescence Intensity
CVD 908-*htrA*	0.98	100	0.6	1.11	100	0.6	1.12	100	0.6
pGEN91	1.00	13.2	28.6	1.17	11.4	42.9	1.26	10.9	65.5
pGEN111	1.26	47.4	51.8	1.17	28.9	93.6	1.12	42.4	65.1
pGEN121	1.01	80.5	53.3	1.20	73.8	74.0	1.15	56.7	105.3
pGEN193	1.11	71.4	50.9	1.24	65.2	64.7	1.22	53.7	90.8
pGEN222	1.01	96.8	52.1	1.28	93.3	67.8	1.13	95.3	89.2

[1]These data are represented as histograms in Figure 8.

[2]All strains were streaked from frozen master stocks onto 2X LB agar supplemented with DHB and 50 μg/ml of carbenicillin, and incubated for 36 hr at 30°C. Isolated colonies were pooled into 300 μl of 1X LB broth supplemented with DHB, from which 25 μl were inoculated into 25 ml of 1X LB broth containing DHB and either 50 mM, 150 mM, or 300 mM NaCl; cultures were incubated at 37°C , 250 rpm for 24 hr. For the results presented in this table, bacteria were then pelleted, resuspended in 1 ml PBS pH 7.4, and then diluted 1:1000 into PBS for analysis by flow cytometry.

In general, as osmolarity increases and induction of P_{ompC1} rises, the percentage of the live vector population expressing GFPuv drops; nevertheless, the mean level of fluorescence intensity increases as expected.. For example, in the presence of 50 mM NaCl, 80.5% of a population of CVD 908-*htrA*(pGEN121) express GFPuv with a mean fluorescence intensity of 53.3. As the concentration of NaCl increases to 300 mM NaCl, the percentage of the population expressing GFPuv drops to 56.7%; nevertheless, the mean fluorescence intensity rises to 105.3. However, it is notable that for strains carrying pGEN222 with a complete plasmid maintenance system (i.e *hok* - *sok* + *par* + *parA*), the percentage of the population expressing the heterologous antigen remains at approximately 95%, while the mean fluorescence intensity increases from 52.1 (50 mM NaCl) to 89.2 (300 mM NaCl). It was noted that upon further passage of these strains for an additional 24 hrs in the absence of antibiotic selection, less than 5% of bacteria continued to express functional GFPuv. Streaks of these cultures onto solid medium, prior to flow analysis, indicated that non-fluorescing bacteria remained viable, but were sensitive to antibiotic selection. When non-fluorescing bacteria were sorted and plated, they were confirmed to be sensitive to antibiotic and non-fluorescent when irradiated with ultraviolet light, indicating loss of resident plasmids.

A passage experiment involving CVD 908-*htrA* carrying expression plasmids with an *ori*101 origin detected no significant loss of GFPuv expression after passage of strains for 48 hrs without selection, regardless of osmolarity. Therefore, strains were passaged in a separate experiment for 96 hrs (i.e. 4 x 24 hr) in the presence of either 50, 150, or 300 mM NaCl. Populations were analyzed by flow cytometry after 3 and 4 passages, and results are recorded in Table 7.

TABLE 7 shows stability within CVD 908-*htrA* of *ori*101 replicons, containing plasmid maintenance systems of increasing complexity, grown without selection and in the presence of increasing osmolarity.

TABLE 7

STRAIN (Passage Number)[1]	50 mM NaCl			150 mM NaCl			300 mM NaCl		
	$O.D._{600}$	Percent Fluorescing Bacteria	Mean Fluorescence Intensity	$O.D._{600}$	Percent Fluorescing Bacteria	Mean Fluorescence Intensity	$O.D._{600}$	Percent Fluorescing Bacteria	Mean Fluorescence Intensity
CVD 908-*htr*A (#3)	ND [2]	100	0.6	ND	100	0.5	ND	100	0.5
CVD 908-*htr*A (#4)	1.00	100	0.3	1.18	100	0.3	1.19	100	0.3
pGEN132 (#3)	ND	45.5	29.0	ND	33.2	36.9	ND	81.3	47.3
pGEN132 (#4)	1.03	10.9	27.8	1.20	7.6	36.1	1.32	51.3	47.5
pGEN142 (#3)	1.05	99.5	35.5	1.23	98.9	45.1	1.28	96.5	47.8
pGEN142 (#4)	1.17	94.4	38.0	1.29	91.5	45.0	1.33	93.9	47.7
pGEN206 (#3)	1.08	98.1	36.2	1.25	94.5	42.8	1.29	95.2	47.4
pGEN206 (#4)	1.13	80.2	32.6	1.26	68.6	36.6	1.33	93.5	41.3

[1]All strains were streaked from frozen master stocks onto 2X LB agar supplemented with DHB and 50 μg/ml of carbenicillin, and incubated for 36 hr at 30°C. Isolated colonies were pooled into 300 μl of 1X LB broth supplemented with DHB, from which 25 μl were inoculated into 25 ml of 1X LB broth containing DHB and either 50 mM, 150 mM, or 300 mM NaCl; cultures were incubated at 37°C, 250 rpm for 24 hr (defined here as passage #1). For passage #2, 25 μl from passage #1 were inoculated into 25 ml (i.e. 1:1000 dilution) of identical medium and incubated at 37°C, 250 rpm for an additional 24 hr without selection. Passages 3 and 4 were carried out in identical fashion, but after the next passage had been set up the remaining bacteria were then pelleted, resuspended in 1 ml PBS pH 7.4, and then diluted 1:1000 into PBS for analysis by flow cytometry.

[2]ND = not done.

Live vectors carrying unstabilized *ori*101 replicons eventually lost the capacity to synthesize the heterologous antigen after 96 hr. For example, after 96 hr growth in the presence of 50 mM NaCl, only 10.9% of CVD 908-*htrA*(pGEN132) expressed GFPuv and fluoresced. As the concentration of NaCl in the medium was increased to 150 mM, fluorescence was detected in only 7.6% of the population; curiously, at 300 mM NaCl, the percentage recovered to 51.3% fluorescing bacteria. Remarkably, CVD 908-*htrA* carrying either pGEN142 (*hok* - *sok*) or

pGEN206 (*hok* - *sok* + *parA*) retained synthesis of GFPuv in greater than 95% of the population after 3 passages (72 hr), regardless of osmolarity (see Table 7). The percentage of fluorescing CVD 908-*htrA* (pGEN142) remained near this level after 4 passages (96 hr), while decreasing slightly for CVD 908-*htrA* (pGEN206).

Taken together, these data show that as copy number is reduced, the apparent stability of resident plasmids and proficiency of a live vector to synthesize a heterologous antigen such as GFPuv increases; as plasmid maintenance systems accumulate within a given plasmid, apparent stability and antigen synthesis are further enhanced. In addition, as the induction of P_{ompC1} and concomitant production of the heterologous antigen increases, the percentage of a growing population remaining capable of synthesizing antigen can be dramatically reduced.

6.7 Bacterial strains and culture conditions

All plasmid constructions were recovered in *Escherichia coli* strain DH5α or DH5αF'IQ (Gibco BRL). Construction of the *hok* - *sok* gene cassette used pR1 template DNA isolated from *E. coli* strain J53(pR1), a generous gift from James B. Kaper. The live vector *S. typhi* CVD 908-*htrA* is an auxotrophic derivative of the wild type strain Ty2 with deletions in *aroC, aroD*, and *htrA* (Tacket *et al.* 1997b). All strains used for examination of plasmid stability were grown in media supplemented with 2,3-dihydroxybenzoic acid (DHB) as previously described (Hone *et al.* 1991; Galen *et al.* 1997). When grown on solid medium, plasmid- bearing strains of CVD 908-*htrA* were streaked from frozen (-70°C) master stocks onto 2X Luria- Bertani agar containing (per liter) 20 g Bacto tryptone, 10 g Bacto yeast extract, and 3 g NaCl (2X LB agar) plus carbenicillin at a concentration of 50 μg/ml. Plates were incubated at 30°C for 24 - 36 hr to obtain isolated colonies ~2mm in diameter; strains were incubated at 30°C to minimize the toxicity of GFPuv expression in CVD 908-*htrA*.

When grown in liquid medium, cultures were incubated at 37°C, 250 rpm for 16 - 24 hr. To examine the osmotic induction of the *ompC* promoter (P_{ompC}) within either *E. coli* DH5α or CVD 908-*htrA*, strains were grown in Bacto nutrient broth (Difco) containing DHB and either NaCl or sucrose; cultures were supplemented either with 50 μg/ml of carbenicillin or increasing concentrations of kanamycin where P_{ompC} - *aphA-2* cassettes were examined. For quantitation of GFPuv synthesis using flow cytometry, 6-8 isolated colonies from master stocks streaked onto 2X LB agar as above were inoculated into 25 ml of 1X LB broth supplemented with 50

μg/ml carbenicillin where desired and NaCl at increasing concentrations to increase the induction of *ompC* promoters. Cultures were incubated at 37°C, 250 rpm for 16 - 24 hr prior to pelleting bacteria for flow cytometry as described below.

6.8 Molecular genetic techniques.

Standard techniques were used for the construction of the plasmids represented here (Sambrook *et al.*, 1989). Unless otherwise noted, native *Taq* DNA polymerase (Gibco BRL) was used in polymerase chain reactions (PCR). *S. typhi* was prepared for electroporation of recombinant plasmids after harvesting from Miller's LB broth (Gibco BRL) supplemented with DHB; after pelleting bacteria, the cells were washed thrice with one culture volume of sterile distilled water and resuspended in sterile distilled water to a final volume of 1/100 of the original culture volume. Electroporation of strains was performed in a Gene Pulser apparatus (Bio-Rad) set at 2.5 kV, 200 Ω, and 25 μF. Following electroporation, bacteria were repaired using SOC medium and incubating at 37°C, 250 rpm for 45 min; bacteria were then plated on 1X LB medium containing DHB plus 50 μg/ml carbenicillin, and incubated at 30°C for 24 hr. Isolated colonies were then swabbed onto supplemented 2X LB and incubated at 30°C for 16 hr. Frozen master stocks were prepared by harvesting bacteria into SOC medium without further supplementation and freezing at -70°C.

6.9 Construction of Expression Vectors

The expression vectors listed in the following Table 8 were prepared in the course of the recent work.

TABLE 8

Plasmid	Size (kb)	Relevant genotype	Reference
pTET*nir*15	3.7	*ori*E1 *toxC* *bla*	Oxer *et al.* (1991)
pJN1	1.9	*ori*E1 *bla*	This work
pJN2	3.4	*ori*E1 *toxC* *bla*	This work
pGFPuv	3.3	pUC19*ori* *gfpuv* *bla*	Clontech
pGFP*ompC*	3.5	*ori*E1 *gfpuv* *bla*	This work
pNRB1	3.5	*ori*E1 *gfpuv* *tetA*	This work
pGEN2	4.2	*ori*E1 *gfpuv* *tetA* *hok-sok*	This work

TABLE 8			
Plasmid	**Size (kb)**	**Relevant genotype**	**Reference**
pGEN3	4.1	*ori*15A *gfpuv tetA hok-sok*	This work
pGEN4	5.6	*ori*101 *gfpuv tetA hok-sok*	This work
pJN5	3.1	*ori*E1 *gfpuv bla*	This work
pJN6	3.7	*ori*E1 *gfpuv bla hok-sok*	This work
pJN7	4.1	*ori*E1 *gfpuv bla hok-sok par*	This work
pJN8	5.4	*ori*E1 *gfpuv bla hok-sok parA*	This work
pGEN51	3.6	*ori*E1 *gfpuv bla*	This work
pGEN71	4.2	*ori*E1 *gfpuv bla hok-sok*	This work
pGEN84	4.5	*ori*E1 *gfpuv bla hok-sok par*	This work
pGEN183	5.9	*ori*E1 *gfpuv bla hok-sok parA*	This work
pGEN211	6.2	*ori*E1 *gfpuv bla hok-sok par parA*	This work
pGEN91	3.5	*ori*15A *gfpuv bla*	This work
pGEN111	4.1	*ori*15A *gfpuv bla hok-sok*	This work
pGEN121	4.5	*ori*15A *gfpuv bla hok-sok par*	This work
pGEN193	5.8	*ori*15A *gfpuv bla hok-sok parA*	This work
pGEN222	6.2	*ori*15A *gfpuv bla hok-sok par parA*	This work
pGEN132	4.8	*ori*101 *gfpuv bla par*	This work
pGEN142	5.4	*ori*101 *gfpuv bla par hok-sok*	This work
pGEN206	7.1	*ori*101 *gfpuv bla par hok-sok parA*	This work

6.9.1 Construction of pJN1 and pJN2

The expression plasmids constructed for these studies are composed of 3 basic cassettes encoding 1] expression of a heterologous antigen, 2] a plasmid origin of replication, and 3] selection and maintenance functions. To accomplish this, a basic replicon was constructed in which these cassettes were separated by unique restriction sites. The primers used in construction of the plasmid cassettes are set forth in the following Table 9:

TABLE 9					
Primer number	**Sequence[1]**	**Cassette created**	**GenBank Accession Number**	**Region of Homology[2]**	**Region of Complementarity[3]**
1	5'-GCAGGAAAGAACATGTGAGCCTAGGGCCAGCAAAAGGCCAGGAAC-3'	*ori*E1	J01749	2463 - 2507	
2	5'-CATGACCAAAATCCCTTAACTAGTGTTTTAGATCTACTGAGCGTCAGACCCCG-3'	"	"		3197 - 3145
3	5'-CGGGGTCTGACGCTCAGTAGATC	*bla*	"	3145 - 3197	

TABLE 9

Primer number	Sequence[1]	Cassette created	GenBank Accession Number	Region of Homology[2]	Region of Complementarity[3]
	TAAAACACTAGTTAAGGGATTTTGG T CATG-3'				
4	5'-GCTGTCAAACATGAGAATTCTAG AAGACGAAAGGGCCTCGTGATACG CC-3'	"	"		17 - 1, 4361 - 4330
5	5'-ACAGCCTGCAGACAGATCTTGAC AGCTGGATCGCACTCTGGTATAATT GGG AAGCCCTGCAAAG-3'	*aphA-2*	V00618	1-64	
6	5'-CGAAGCCCAACCTTTCATAGAAG CTAGCGGTGGATCCGAAATCTCGT GAT GGCAGGTTG-3'	"	"		1044 - 986
7	5'-AACAAGCGTTATAGGAATTCTGT GGTAGCA-3'	P_{ompC}	K00541	4 - 33	
8	5'-ACTTTCATGTTATTAAAGATCTGT TATATG-3'	"	"		498 - 469
9	5'-AGATCTTAATCATCCACAGGAGG CTTTCTGATGAGTAAAGGAGAAGAA C TTTTCACTGG-3'	*gfpuv*	U62636	289 - 317	
10	5'-GCTAGCTCATTATTTGTAGAGCTC ATCCATGC-3'	"	"		1008 - 983
11	5'-AGATCTGAATTCTAGATCATGTTT GACAGCTTATCATCGATAAGCTTTA ATGCG-3'	*tetA*	J01749	4 - 41	
12	5'-AGATCTTATCAGGTCGAGGTGGC CCGGCTCCATGCACCGCGACGCAA CG CG-3'	"	"		1275 - 1234
13	5'-CGCGAATTCTCGAGACAAACTCC GGGAGGCAGCGTGATGCGGCAACA A TCACACGGATTTC-3'	*hok-sok-tetA*	X05813	2 - 48	
14	5'-ATGAGCGCATTGTTAGATTTCATT TTTTTTTCCTCCTTATTTTCTAGACA A CATCAGCAAGGAGAAAGG-3'	"	J01749, X05813		108 - 86, 580 - 559
15	5'-CCTTTCTCCTTGCTGATGTTGTCT AGAAAATAAGGAGGAAAAAAAAATG AAATCTAACAATGCGCTCAT-3'	"	X05813, J01749	559 - 580, 86 - 108	
16	5'-GCTACATTTGAAGAGATAAATTGC ACTGGATCCTAGAAATATTTTATCTG ATTAATAAGATGATC-3'	*ori*15A	X06403		1461 - 1397
17	5'-CGGAGATTTCCTGGAAGATGCCT AGGAGATACTTAACAGGGAAGTGA GAG-3'	"	"	780 - 829	
18	5'-GTCTGCCGGATTGCTTATCCTGG CGGATCCGGTTGACAGTAAGACGG GTAAGCCTGTTGAT-3'	*ori*101	X01654	4490 -4550	
19	5'-CCTAGGTTTCACCTGTTCTATTAG GTGTTACATGCTGTTCATCTGTTAC ATTGTCGATCTG-3'	"	"		6464 - 6408
20	5'-AGGCTTAAGTAGCACCCTCGCAA GATCTGGCAAATCGCTGAATATTCC TTTTGTCTCCGAC-3'	*par*	X01654		4918 - 4858

TABLE 9

Primer number	Sequence[1]	Cassette cr ated	GenBank Accession Number	Region of Homology[2]	Region of Complementarity[3]
21	5'-GAGGGCGCCCCAGCTGGCAATT CTAGACTCGAGCACTTTTGTTACCC GCCAAACAAAACCCAAAAACAAC-3'	*aphA2- pa rA*	V00618, X04268	38 - 16, 1 - 37	
22	5'-AGAAGAAAAATCGAATTCCAGCA TGAAGAGTTTCAGAAAATGACAGAG CGTGAGCAAGTGC-3'	"	X04268		1704 - 1644
23	5'-CGAAGCCCAACCTTTCATAGAAA CTAGTGGTGGAATCGAAATCTCGTG ATGGCAGGTTG-3'	"	V00618		1044 - 986
24	5'-GTTGTTTTTGGGTTTTGTTTGGCG GGTAACAAAAGTGCTCGAGTCTAGA ATTGCCAGCTGGGGCGCCCTC-3'	"	X04268, V00618	37 - 1, 16 - 38	

[1]Relevant restriction sites are underlined and referred to in the text; ribosome binding sites and start codons are designated in *italics*.

[2]Refers to the sequence within the coding strand of a given gene, to which the primer is homologous.

[3]Refers to the sequence within the **non**-coding strand of a given gene, to which the primer is homologous.

pTET*nir*15 (see Table 8; Oxer *et al.* 1991) was re-engineered such that the *ori*E1 origin of replication and *bla* gene were separated by a unique *Spe*I site. Toward this end, an *ori*E1 cassette was synthesized by PCR using Vent polymerase with primers 1 and 2 and pCVD315 (Galen *et al.* 1990) as the template. The resulting 735 bp fragment carries engineered *Spe*I and *Bgl*II sites 5'-proximal to the promoter controlling transcription of RNA II, and an engineered *Avr*II site 675 bases from these sites. A separate PCR reaction was carried out using primers 3 and 4 to create a 1234 bp *bla* cassette containing an engineered *Xba*I site 5'-proximal to the original *Eco*RI site. The products from these two PCR reactions were gel purified and used in an overlapping PCR with primers 1 and 4 to yield a final 1916 bp *ori*E1 - *bla* fragment which was self-ligated to create pJN1. The P_{nir15} -*toxC* fragment from pTET*nir*15 was excised as an *Eco* RI (partial digestion)- *Ava*I fragment, in which the *Ava*I terminus was polished, and inserted into the multiple cloning region from pSL1180 (Brosius, 1989) cleaved with *Eco* RI and *Stu*I; this cassette was then re-excised as an *Eco* RI (partial digestion)- *Avr*II fragment and inserted into pJN1 cleaved with *Eco* RI - *Avr*II, creating pJN2 (see Table 8).

6.9.2 C nstruction of pGFP*ompC*

To facilitate screening of a functional osmotically regulated P_{ompC} allele from *Escherichia coli*, an *aphA-2* cassette was constructed, encoding resistance to the aminoglycosides neomycin and kanamycin (Shaw *et al.* 1993). A polymerase chain reaction (PCR) was carried out using primers 5 and 6 with the template pIB279 (Blomfield *et al.* 1991) to generate a 1044 bp product, from which a promoterless 903 bp *aphA-2* *Bgl*II-*Nhe*I fragment was cleaved for replacement of a *Bgl*II-*Nhe*I *toxC* cassette encoding fragment C of tetanus toxin in pTET*nir*15. The anaerobically regulated P_{nir15} promoter was replaced with a 459 bp *Eco*RI-*Bgl*II P_{ompC} allele constructed using primers 7 and 8 with chromosomal template DNA from *E. coli* DH5α to create pK*ompC*. After confirming osmotic induction of P_{ompC} by examining the increase in resistance to kanamycin with increasing osmolarity, the *aphA-2* cassette was then replaced with a *gfpuv* gene encoding a prokaryotic codon-optimized GFPuv allele (Clontech; Crameri *et al.* 1996). The *gfpuv* gene was recovered by PCR using primers 9 and 10 with the template pGFPuv to generate a 751 bp *Bgl*II-*Nhe*I fragment which was inserted into pK*ompC*, to generate pGFP*ompC*. Colonies were screened for functional GFPuv, and the brightest colonies were then examined for induction of fluorescence with increasing concentrations of NaCl. A P_{ompC1} - *gfpuv* cassette was cleaved from pGFP*ompC1* as an *Eco*RI - *Nhe*I fragment and inserted into a derivative of pJN2 cleaved with *Eco*RI - *Nhe*I to create pJJ4.

6.9.3 Construction of pNRB1, pGEN2, pGEN3, and pGEN4

Since it was intended that copy number not be influenced by transcription originating from promoters outside the origin of replication, it was necessary to ensure that all replication cassettes were flanked at both ends by transcription terminators. Because the origin and antigen cassettes of pJN2 are separated by the *trpA* terminator, it was only necessary to insert one additional terminator between the origin and *bla* cassettes.

To facilitate construction of additional plasmids later on, a *tetA*-T1T2 cassette was created. pYA292 (Galan *et al.* 1990) was first cleaved with *Hind*III and *Bgl*II, and the T1T2 terminator fragment was polished and inserted into the *Sma*I site of the pBluescript II KS (Stratagene) mutiple cloning region; when the proper orientation was identified, this cassette was re-excised as a *Bam*HI- *Pst*I fragment and inserted into pIB307 (Blomfield *et al.* 1991) cleaved with

*Bam*HI-*Pst*I, creating pJG14. It was later determined by sequence analysis that the cassette had undergone a deletion of approximately 100 bp, removing half of the T2 terminator.

Using pBR322 as a template, primers 11 and 12 were used to synthesize a 1291 bp *tetA Bgl*II fragment. This *tetA Bgl*II fragment was then inserted into the *Bam*HI site of pJG14 such that transcription of the *tetA* gene is terminated at the T1T2 terminator, creating pJG14*tetA*. Finally, this *tetA*-T1T2 cassette was cleaved from pJG14*tetA* as an *Eco*RI -*Pst*I fragment in which the *PstI* site had been removed by polishing; the resulting fragment was inserted into pJJ4, cleaved with *Spe*I, polished, and recleaved with *Eco*RI to replace the *bla* cassette and create pNRB1.

The non-catalytic post-segregational killing function to be incorporated into the plasmid maintenance systems of the expression plasmids described here was the *hok-sok* locus, from the multiple drug resistance R-factor pR1. Initial attempts at recovering the *hok-sok* locus after PCR were unsuccessful. It was therefore necessary to use overlapping PCR to generate a cassette in which *hok-sok* was transcriptionally fused to a promoterless *tetA* gene such that transcription originating from the *hok* promoter would continue into *tetA* and result in a transcript encoding both Hok and resistance to tetracycline. pR1 plasmid DNA was purified from *E. coli* J53(pR1) in which pR1 encodes resistance to both carbenicillin and chloramphenicol. A 640 bp *hok-sok* fragment was synthesized using primers 13 and 14; a promoterless 1245 bp *tetA* fragment was recovered in a separate PCR using primers 15 and 12 with pNRB1 as the template. The products from these two PCR reactions were then used in an overlapping PCR with primers 12 and 13 to yield the final 1816 bp *hok-sok-tetA* fragment. This fragment was inserted as an *Eco*RI-*Sph*I fragment into pNRB1 cleaved with *Eco*RI-*Sph*I, regenerating the *tetA* gene and creating pGEN1.

A set of 3 isogenic plasmids was then constructed, differing only in copy number, from which all further expression plasmids would be derived. The *Bgl*II-*Avr*II origin of replication cassette of pGEN1 was replaced by a *Bgl*II-*Avr*II *ori*E1 cassette from pJN2 to generate pGEN2. An *ori*15A replication cassette was synthesized by PCR using primers 16 and 17 with pACYC184 template to generate a 629 bp *Bam*HI-*Avr*II fragment, which was inserted into pGEN2 cleaved with *Bgl*II-*Avr*II to create pGEN3. Finally, an *ori*101 replication cassette was synthesized by PCR using primers 18 and 19 with pSC101 template, generating a 1949 bp *Bam*HI-*Avr*II fragment which was inserted into pGEN2 cleaved with *Bgl*II-*Avr*II to create pGEN4.

6.9.4 Construction of pJN5, pGEN51, pGEN91, and pGEN132

The principle set of isogenic expression plasmids, to which individual elements of a plasmid maintenance system were sequentially added, was composed of pGEN51 (containing *ori*E1), pGEN91 (containing *ori*15A), and pGEN132 (containing *ori*101). The basic replicon from which these 3 plasmids were constructed was pJN5, which was assembled by cleaving the P_{ompC} - *gfpuv* cartridge as an *Eco*RI-*Nhe*I fragment from pGFP*ompC* to replace the P_{nir15} -*toxC* cassette of pJN2. Construction of pGEN51 was then accomplished by removal of the replication cassette from pGEN2 as a *Bam*HI fragment, and replacement of the origin of replication within pJN5 digested with *Bgl*II and *Bam*HI, thereby regenerating the *gfpuv* gene. Construction of pGEN91 and pGEN132 were constructed in an identical manner by excision of origin cassettes as *Bam*HI fragments from pGEN3 and pGEN4 respectively (see Figure 7 for representation of isogenic expression plasmids based on pGEN91).

6.9.5 Construction of pJN6, pGEN71, pGEN111, and pGEN142

The *hok-sok* locus was then inserted as an *Xba*I -*Sal*I fragment into pJN5 cleaved with *Xba*I and *Sal*I, again regenerating the *gfpuv* gene to create pJN6 (see Table 2). Construction of pGEN71, pGEN111, and pGEN142 was then carried out exactly as for pGEN51, pGEN91, and pGEN132 by insertion into pJN6 of origin cassettes as *Bam*HI fragments from pGEN 2, pGEN3, and pGEN4 respectively.

6.9.6 Construction of pJN7, pGEN84, and pGEN121

Construction of *ori*E1 and *ori*15A expression plasmids containing a plasmid maintenance system, composed of both a post- segregational killing system and at least one partition function, was first attempted using the *par* function from pSC101. A 377 bp *Bam*HI-*Bgl*II fragment was synthesized using primers 18 and 20 with pSC101 template DNA; this fragment was inserted into pJN6 cleaved with *Bgl*II to create pJN7. As in the constructions above, origin cassettes from pGEN2 and pGEN3 were then excised as *Bam*HI fragments and inserted into pJN7 digested with *Bgl*II and *Bam*HI to create pGEN84 and pGEN121.

6.9.7 Construction of pJN8, pGEN183, pGEN193, pGEN206, pGEN211 and pGEN222

The final expression plasmids were constructed by introduction of the *parA* active partitioning locus from pR1. As with *hok-sok*, initial attempts at recovering the *parA* locus after PCR were unsuccessful. It was necessary to use overlapping PCR to generate an *aph-parA* cassette, in which *aph* and *parA* were divergently transcribed and separated by *Xba* I and *Xho*I sites, to enable subcloning of the *parA* locus. A 1737 bp *parA* fragment was synthesized using primers 21 and 22 with pR1 template; a 1076 bp *aphA-2* fragment was recovered in a separate PCR using primers 23 and 24 with pIB279 as the template. The products from these two PCR reactions were then used in an overlapping PCR with primers 22 and 23 to yield the final 2743 bp *aphA2-parA* fragment. This fragment was inserted as a 2703 *Eco*RI-*Spe*I fragment into pJN6. The *parA* cassette was then re- excised as an *Xho*I fragment and inserted again into pJN6 cleaved with *Xho*I, regenerating the *gfpuv* gene, and creating pJN8.

Plasmids carrying a plasmid maintenance system composed of the post-segregational killing *hok-sok* function and *parA*, were constructed by excision of *ori*E1 and *ori*15A *Bam*HI-*Spe*I cassettes from pGEN51 and pGEN91 respectively, and insertion into pJN8 cleaved with *Bam*HI and *Spe*I to create pGEN183 and pGEN193 respectively. Plasmids containing the full complement of *hok-sok*, *par*, and *parA* maintenance functions were constructed by insertion of *par*-containing origin cassettes as *Bam*HI-*Spe*I cassettes from pGEN84, pGEN121, and pGEN132 into pJN8 cleaved with *Bam*HI and *Spe*I to create pGEN211, pGEN222, and pGEN206 respectively.

6.10 Quantitation of GFPuv and Plasmid Maintenance

Quantitation of GFPuv and plasmid maintenance were analyzed by measuring the fluorescence of plasmid-bearing live vectors using an Epics Elite ESP flow cytometer/cell sorter system (Coulter) with the argon laser exciting bacteria at 488 nm and emissions detected at 525 nm. 25 ml 1X LB cultures grown as described above were pelleted, and bacteria were resuspended into 1 ml of PBS. Cells were then diluted 1:1000 into PBS prior to determination of viable counts and flow analysis. Forward versus side light scatter, measured with logarithmic amplifiers, was used to gate on bacteria. A minimum of 50,000 events were acquired from each sample at a collection rate of approximately 3500 events per second. Mean fluorescence intensity for a given bacterial population was determined using the Epics Elite Software Analysis

Package. The levels of autofluorescence, determined using plasmidless *S. typhi* CVD 908-*htrA* and *E. coli* DH5α strains, were used to place markers quantitating the percentages of bacteria in a given population expressing GFPuv.

6.11 Conclusions

The broad objective of the research presented in Sections 6.6 – 6.10 was to investigate the feasibility of developing a plasmid maintenance system for the stabilization of multicopy expression plasmids encoding foreign antigens in an *S. typhi* live vector vaccine strain, without additional modification of the chromosome. The maintenance of expression plasmids was enhanced at two independent levels. First, dependence upon balanced-lethal maintenance systems that involve catalytic enzymes expressed from multicopy plasmids was removed; this was accomplished through incorporation into expression plasmids of a post-segregational killing system based on the non-catalytic *hok-sok* plasmid addiction system from the antibiotic-resistance factor pR1. At least one naturally occurring plasmid partition function was also introduced into these expression plasmids, to potentially eliminate random segregation of such plasmids, thereby enhancing their inheritance and stability.

Although these expression plasmids are ultimately intended to express immunogenic and protective antigens for delivery to the human immune system, GFPuv was selected as a test reporter antigen because quantitation of mean fluorescence in a population of growing live vectors could be used as a measure of the stability of resident plasmids within the live vector. All expression plasmids carried an identical antigen expression cassette, with a P_{ompC1} allele controlling transcription, and translation optimized by incorporation of a consensus ribosome binding site. Because no catalytic activity is associated with the fluorescence of GFPuv, the level of fluorescence intensity measured by flow cytometry within individual bacteria could be correlated directly with gene dosage and copy number. In addition, use of an osmotically regulated *ompC* promoter allowed an assessment of plasmid stability and live vector viability as increasing osmolarity induced higher levels of GFPuv synthesis and presumably higher levels of metabolic stress on the live vector. As seen in Table 2, we confirmed that the P_{ompC1} allele engineered for these studies was responsive to increased osmolarity; when driving expression of an *aph-2* resistance gene, resistance to less than 50 μg/ml kanamycin was observed in the absence of osmotic pressure but resistance increased to greater than 800 μg/ml in the presence of 300 mM NaCl. It was surprising that although the P_{ompC1} allele was engineered

from the chromosomal locus of *E. coli*, it appeared to function more efficiently in *S. typhi*. The uninduced level of expression of GFPuv was the same for both DH5α and CVD 908-*htrA* (mean fluorescence intensity of 4.45 vs 5.37 respectively, Table 3). However, GFPuv synthesis increased 70% in DH5α after induction, but rose over 300% in CVD 908-*htrA* (mean fluorescence intensity of 7.69 vs 23.4 respectively). This effect was not limited to the P_{ompC1} allele but was equally remarkable when using P_{ompC3} (Table 3). These data do not agree with recent observations of Martinez-Flores *et al* (1999) who reported that *E. coli ompC -lacZ* genetic fusions expressed constitutively within *S. typhi*, and that this constitutive level of expression was comparable to induced levels within *E. coli*. Although we have identified a defined locus of point mutations at the 3'-terminus of our *E. coli* P_{ompC1} allele which could explain its osmotically controlled behavior within *S. typhi* CVD 908-*htrA*, such mutations were not identified within P_{ompC3}, which also responds to osmolarity within CVD 908-*htrA*. It should be noted, however, that the genetic fusions studied by Martinez-Flores *et al* involved 1,150 bp of the *E. coli* 5' *ompC* upstream control region, while the P_{ompC} alleles constructed here involve only 459 bp of the 5'-proximal control region of *ompC*. Regardless of this discrepancy, it is encouraging that the highest levels of regulated heterologous gene expression are observed within the attenuated *S. typhi* live vector vaccine strain.

The contributions of several plasmid maintenance systems to the stability of plasmids within CVD 908-*htrA*, growing in the absence of antibiotic selection, were then examined. No combination of maintenance functions could stabilize plasmids containing *ori*E1 origins of replication; in fact, these constructs were difficult to propagate even in the presence of antibiotic. These observations cast doubt upon the rationale for using higher copy number plasmids to optimize expression of heterologous antigens within the cytoplasm of *S. typhi*-based live vectors, a strategy that, heretofore, has been followed by other groups investigating *Salmonella* as live vectors (Covone *et al.* 1998).

Incorporation of plasmid maintenance systems into plasmids carrying an *ori*15A origin of replication was more encouraging. When live vectors carrying such plasmids were passage without selection for 24 hr at 37°C, the effects of various combinations of maintenance functions became apparent. In the absence of maintenance functions, the *ori*15A replicon pGEN91 was lost from greater than 90% of the population, regardless of the level of induction of P_{ompC1} (see Table 6 and Figure 8). With incorporation of the *hok - sok* post-segregational killing locus in pGEN111, the percentage of bacteria expressing GFPuv tripled under all

induction conditions, confirming the observations of others that the *hok* - *sok* locus enhances the stability of *ori*15A replicons (Gerdes *et al.* 1985; Gerdes, 1988; Gerdes *et al.* 1997b). However, it was still noted that regardless of induction conditions, greater than 50% of the bacterial population no longer fluoresced. Since it was confirmed that at least a portion of this non-fluorescing population was still viable and lacked drug resistance, these data confirm previous reports (Gerdes *et al.* 1986; Wu and Wood, 1994; Pecota *et al.* 1997) that the presence of a *hok* - *sok* post-segregational killing system is insufficient by itself to ensure that plasmidless viable bacteria will not arise in a growing population.

One possible mechanism that allows for escape from the influence of *hok* - *sok* involves spontaneous point mutations arising within the lethal Hok open reading frame, which could conformationally inactivate Hok and thereby allow plasmid loss to occur without lethality. This point emphasizes the requirement of multiple mechanisms for enhancing the stability of resident plasmids within growing bacteria; should one maintenance function become inactivated, the probability of other independent functions simultaneously becoming inactivated becomes vanishingly small. Indeed, such redundancy in maintenance functions is widespread within naturally occurring low copy number plasmids (Nordstrom and Austin, 1989). For example, the *Escherichia coli* sex factor F contains one active partitioning function (*sop*) and two killing systems (*ccd* and *flm*) (Loh *et al.* 1988; Golub and Panzer, 1988; Van Melderen *et al.* 1994; Niki and Hiraga, 1997). Similarly, the drug resistance plasmid pR1 contains the active partitioning function *parA*, as well as the post-segregational killing system *hok* - *sok*; in addition, it carries yet another recently defined *kis* - *kid* killing system (Bravo *et al.* 1987; Bravo *et al.* 1988; Ruiz-Echevarria *et al.* 1995). We demonstrate in work reported here that insertion into multicopy *ori*15A replicons of a more complete maintenance system, composed of both a post-segregational system and two partition functions, dramatically improves the stability of these expression plasmids in the absence of selection, regardless of induction conditions for heterologous antigen expression. However, after passage without selection for 48 hrs, plasmids were eventually lost from the bacterial population, due to escape from the lethality of Hok. This problem has recently been addressed by Pecota *et al* (1997) who reported that incorporation of dual killing systems significantly improved plasmid stability when compared to the use of *hok- sok* alone; no partition functions were present in these plasmids. Perhaps inclusion of the *kis* - *kid* killing system, to more fully represent the complement of pR1 stability functions, may be required for optimal stability of higher copy expression plasmids within *S. typhi* live vectors; since *phd-doc* PSK cassettes have recently been constructed, we are also

examining the compatibility of this PSK function in our expression plasmids pGEN211, pGEN222 and pGEN206.

A comparison of strains carrying pGEN121 (an *ori*15A replicon carrying *hok* - *sok* + *par*; ~15 copies per chromosomal equivalent) with the much lower copy number plasmid pGEN142 (an *ori*101 replicon carrying *hok* - *sok* + *par*; ~5 copies per chromosomal equivalent) shows that under conditions of maximum induction of P_{ompC1} with 300 mM NaCl, 57% of a population of CVD 908- *htrA*(pGEN121), passaged for only 24 hr without selection, fluoresce with a mean fluorescence intensity of 105.3; for a population of CVD 908-*htrA*(pGEN142), passaged for 96 hr without selection under identical induction conditions, 94% of the bacteria analyzed by flow cytometry still maintain a mean fluorescence intensity of 47.7. Based on such results with GFPuv as a test antigen, it is tempting to speculate that an optimum level of heterologous antigen presented by an attenuated *S. typhi*-based live vector vaccine to the human immune system can be achieved by decreasing the copy number of resident expression plasmids to perhaps 5 copies per chromosomal equivalent.

The efficiency of eliciting an immune response directed against a heterologous antigen will depend in part upon the ability of the live vector to present such antigens to the immune system. The ability of a live vector to present antigens will in turn depend upon the stability of multicopy expression plasmids that encode the heterologous antigens. Our results demonstrate that inclusion of a plasmid maintenance system within multicopy expression plasmids, without further genetic manipulation of the live vector, enhances the stability of such expression plasmids. However, the presence of multicopy plasmids may also influence the metabolic fitness of the live vector. This is relevant because some foreign antigens of interest exert a deleterious effect on the live vector.

While we do not intend to be bound to this theory, we conclude that a significant metabolic burden is placed upon CVD 908-*htrA* carrying a multicopy expression plasmid; as copy number and/or level of gene expression increases, metabolic burden increases. Studies with *E. coli* have clearly established that plasmid-bearing bacteria grow slower than plasmidless bacteria (Boe *et al.* 1987; McDermott *et al.* 1993; Wu and Wood, 1994; Pecota *et al.* 1997; Summers, 1998). It has also been demonstrated that as copy number increases, the growth rate of such strains decreases; similarly, as induction of heterologous genes increases, growth rate decreases further (Wu and Wood, 1994; Pecota *et al.* 1997). Clearly, spontaneous plasmid

loss would remove any metabolic burden and allow plasmidless bacteria to quickly outgrow the population of plasmid-bearing bacteria. In elegant studies, Wu and Wood (Wu and Wood, 1994) showed that plasmid-bearing *E. coli* strains maintained plasmids under conditions where cloned gene expression was low for 100 hr when passaged in the absence of selection; in contrast, under maximum induction conditions, complete plasmid loss occurred within 10 hr. Interestingly, when the *hok- sok* locus was inserted into these expression plasmids, the plasmids were maintained for 300 hr. under uninduced conditions and 30 hr. under inducing conditions. Such a shift in antigen expression within a population of live vector bacteria would be expected to reduce the efficiency of stimulating any immune response specific to the foreign antigen. Our analysis leads us to conclude that the goal for an effective multivalent *S. typhi*-based live vector vaccine is to optimize viability using stabilized lower copy number expression vectors, capable of expressing high levels of heterologous antigen in response to an environmental signal likely to be encountered *in vivo* after the vaccine organisms have reached an appropriate ecological niche. We are currently testing this strategy using the murine intranasal model to examine the immunogenicity of fragment C of tetanus toxin expressed within CVD 908-*htrA* from our expression vectors pGEN211 (*ori*E1), pGEN222 (*ori*15A), and pGEN206 (*ori*101), all of which carry identical plasmid maintenance systems and differ only in copy number. The work presented herein enables the development of single dose, oral *S. typhi*-based live vector vaccines capable of inducing protective immune responses against multiple unrelated human pathogens.

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